# THE EFFECT OF *STREPTOCOCCUS MUTANS* ON THE GROWTH AND VIRULENCE EXPRESSION OF *CANDIDA ALBICANS* IN A MIXED-SPECIES BIOFILM

By

**WANG, XUELING**

A dissertation submitted to the Department of Pre-Clinical Sciences,

M. Kandiah Faculty of Medicine and Health Sciences,

Universiti Tunku Abdul Rahman,

in partial fulfillment of the requirements for the degree of

Master of Medical Science

August 2023

## ABSTRACT

**THE EFFECT OF *STREPTOCOCCUS MUTANS* ON THE GROWTH AND VIRULENCE EXPRESSION OF *CANDIDA ALBICANS* IN A MIXED-SPECIES BIOFILM**

**Wang, Xueling**

*Candida albicans* is a type of fungus commonly found in the oral cavity and various other parts of the human body. While it generally coexists peacefully with the host, under certain circumstances, such as immunosuppression or treatments like chemotherapy and radiotherapy, it can cause invasive lesions and infections. In individuals with weakened immune systems, *C. albicans* has the potential to become pathogenic and penetrate the body's defenses. This can occur due to conditions like HIV/AIDS, organ transplantation, or prolonged use of immunosuppressive medications. Similarly, cancer patients undergoing chemotherapy or radiation therapy often experience a compromised immune system, making them susceptible to *Candida*-related complications. In the oral cavity, *C. albicans* naturally reside in harmony with other microorganisms, forming part of the normal oral flora. However, when the balance is disrupted, such as through a decrease in saliva production, poor oral hygiene, or the use of certain medications (such as broad-spectrum antibiotics), *C. albicans* can overgrow and lead to oral candidiasis, also known as thrush. This condition manifests as white patches or sores on the tongue, inner cheeks, or the back of the throat, causing discomfort, pain, and difficulty in swallowing. Invasive *Candida* infections can extend beyond the oral cavity and affect other areas of the body as well. For individuals with weakened immune systems, *Candida* can

invade the bloodstream, leading to a serious condition known as candidemia. This can result in widespread systemic infections, affecting vital organs such as the heart, kidneys, liver, and brain. If left untreated, candidemia can be life-threatening.

*Streptococcus mutans* is a significant pathogenic bacterium known for its ability to cause dental caries, commonly referred to as tooth decay. In the oral cavity, it forms biofilms, which are complex microbial communities that adhere to various surfaces. Interestingly, *C. albicans* is another prominent member of the oral microbiota that coexists with *S. mutans* within these biofilms. The coexistence of *C. albicans* and *S. mutans* in oral biofilms is of great importance. These biofilms provide a favorable environment for the growth and survival of both microorganisms, enabling them to thrive and contribute to the development of oral diseases. *S. mutans* is particularly adept at utilizing dietary sugars and producing acids as metabolic byproducts. These acids can demineralize the tooth enamel, leading to the formation of cavities. Moreover, *S. mutans* possess unique adhesive properties, allowing them to adhere to the tooth surfaces and facilitate the establishment of biofilms. The presence of this bacterium in the biofilm community greatly enhances its cariogenic potential. *C. albicans*, on the other hand, can form hyphae, which are long, filamentous structures that aid in its attachment to oral surfaces. Within biofilms, *C. albicans* can interact with *S. mutans* and other microorganisms through complex interactions. These interactions can influence the overall structure and composition of the biofilm community, potentially impacting its pathogenicity.

The primary objective of this research endeavor was to investigate the potential influence of *S. mutans* on the virulence and growth patterns of *C. albicans* within mixed-species biofilms, where both organisms coexist. By conducting this study, valuable insights could be gained regarding the potential correlation between caries caused by *S. mutans* and an increased susceptibility to *C. albicans* infections. Understanding such a relationship could have significant implications for patient care and treatment strategies in dental medicine.

Six-well tissue culture plates were utilized to culture both single-species biofilms of *Candida albicans* and mixed-species biofilms consisting of *C. albicans* and *Streptococcus mutans* for a period of 24 hours. The viability of these biofilms was measured by determining the colony-forming units (CFU) per milliliter in single biofilms of *C. albicans* and mixed-species biofilms of *C. albicans* and *S. mutans* with different ratios (*C. albicans*: *S. mutans* = 1:10 and 1:3), while the expression of virulence genes was quantified using RT-qPCR assays. The target genes include four virulence genes: HWP1, EFG1, ALS3, and ACT1, as well as two quorum sensing genes, CHK1 and PBS2. To investigate the impact of different ratios of *C. albicans* and *S. mutans* on biofilm formation, the CFU counts were compared among the mixed and single-species biofilms grown with varying concentrations of *S. mutans*.

The findings of the study indicated that the presence of *S. mutans* did not have a detrimental impact on the expression of specific virulence and quorum sensing genes in *C. albicans*. However, intriguingly, it was observed that the replication of *C. albicans* within a biofilm was significantly enhanced in the presence of *S. mutans*, specifically at a 1:3 ratio of *C albicans* to *S mutan (*p-value <0.05). However, at a 1:10 ratio of *C albicans* to *S mutans,* there was no statistically

significant difference in the colony-forming units (CFU) between single *C. albicans* biofilms and mixed-species biofilms.

These results suggest a potential interaction between the two species that promotes the growth of *C. albicans* within a biofilm environment. To gain a more comprehensive understanding of this phenomenon, further investigations are warranted. It is important to delve deeper into the intricate dynamics of virulence expression in mixed-species biofilms, considering diverse ratios of *C. albicans* and *S. mutans*, as well as varying durations of incubation. By examining these factors, we can shed more light on the interplay between these two species and the influence of their interactions on biofilm formation and pathogenicity. Additional studies will provide valuable insights into the complex relationship between *C. albicans* and *S. mutans*, potentially uncovering novel mechanisms of biofilm development and pathogenicity. Such knowledge could pave the way for targeted therapeutic strategies aimed at disrupting or controlling the growth and virulence of these microorganisms in biofilm-associated infections.

## ACKNOWLEDGEMENT

I would like to express my thanks and gratitude to:

(a) My supervisor, Prof. Yap Sook Fan, for her guidance, advice, and support throughout the entire duration of my study

(b) My co-supervisor, Prof. Ngeow Yun Fong, for her support, patience, and guidance

(c) Fellow students and staff of the Pathogen laboratory, FMHS, who helped and supported me in my research.

(d) UTAR, specifically UTAR Research Fund (UTARRF): IPSR/RMC/UTARRF/ 2020-C2/NO.3

I would also like to thank my family, and my best friends, who support me emotionally in the toughest time.

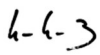
**APPROVAL SHEET**

This dissertation entitled **"THE EFFECT OF *STREPTOCOCCUS MUTANS* ON THE GROWTH AND VIRULENCE EXPRESSION OF *CANDIDA ALBICANS* IN A MIXED-SPECIES BIOFILM"** was prepared by WANG, XUELING and submitted as partial fulfillment of the requirements for the degree of Master of Medical Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Prof. Dr. Yap Sook Fan) Date: 9/Aug/2023
Professor/Supervisor
Department of Pre-clinical Sciences
M. Kandiah Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman



_____

(Prof. Dr. Ngeow Yun Fong) Date: 9/Aug/2023
Professor/Co-supervisor
Department of Pre-clinical Sciences
M. Kandiah Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman

**M. KANDIAH FACULTY OF MEDICINE AND HEALTH SCIENCES**

**UNIVERSITI TUNKU ABDUL RAHMAN**

Date: 9 AUGUST 2023

**SUBMISSION OF DISSERTATION**

It is hereby certified that **WANG, XUELING** (ID No: **20UMM06429**) has completed this dissertation entitled "The Effect of Streptococcus Mutans on the Growth and Virulence Expression of *Candida albicans* in a Mixed-species Biofilm" under the supervision of Prof. Dr. Yap Sook Fan from the Department of Pre-Clinical Sciences, M. Kandiah Faculty of Medicine and Health Sciences, and Prof. Dr. Ngeow Yun Fong from the Department of Pre-Clinical Sciences, M. Kandiah Faculty of Medicine and Health Sciences.

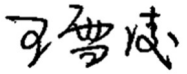
I understand that the University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

(WANG, XUELING)

## DECLARATION

I, WANG, XUELING, hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.



Name: WANG, XUELING

Date: 9 AUGUST 2023

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AHL | N-acyl homoserine lactones |
| AIP | Auto-inducing peptides |
| BHI | Brain Heart Infusion |
| CFU | Colony forming units |
| EPS | Extracellular polymeric substance |
| FBS | Fetal Bovine Serum |
| MAPK | Hog1-activated protein kinase |
| McF | McFarland |
| NTC | No template control |
| PBS | Phosphate buffered saline |
| QS | Quorum sensing |
| RT-qPCR | Reverse Transcription quantitative Polymerase Chain Reaction |
| SDA | Sabouraud Dextrose Agar |
| SDB | Sabouraud Dextrose Broth |
| TCS | Two-component signal transduction system |
| TSB | Trypticase Soy broth |
| ROS | Reactive Oxygen Species |

# CHAPTER 1

# INTRODUCTION

## Background

*C. albicans* is a microorganism that can inhabit various parts of the human body but becomes a pathogen in susceptible individuals. Under normal circumstances, it coexists peacefully with the host, without causing any harm. However, in certain individuals with weakened immune systems or underlying health conditions, *C. albicans* can transform into a pathogenic form and cause invasive infections. Susceptible individuals, such as those with immunodepression, HIV/AIDS, undergoing radiotherapy, or having diabetes, are particularly vulnerable to *C. albicans* infections. These infections can range from mild mucosal infections, such as oral thrush or vaginal yeast infections, to severe and life-threatening systemic infections, including candidemia and invasive candidiasis. One of the key factors contributing to the pathogenicity of *C. albicans* is its ability to transition between different morphological forms, primarily from yeast cells to filamentous hyphae. This transition is often triggered by specific environmental cues, such as changes in temperature, nutrient availability, pH, and host factors. The formation of hyphae allows *C. albicans* to penetrate and invade host tissues. Hyphae possess a filamentous structure with elongated cells that can extend and penetrate host cell membranes. This invasive capability enables *Candida albicans* to gain access to deeper tissues, evade immune responses, and acquire nutrients for its growth and survival.

Biofilm formation is a crucial mechanism by which *C. albicans*, apart from its ability to change its morphology, establishes virulence and invades host tissues. In addition to biofilm formation, *C. albicans* utilizes various other mechanisms to exhibit virulence and cause damage to the host. One significant aspect of *C. albicans* virulence is the production of adhesins. Adhesins are molecules on the surface of the fungal cells that facilitate their attachment to host tissues, enabling colonization and subsequent infection. These adhesins promote adherence to specific host receptors and contributes to the establishment of infection by facilitating the initial interaction between the fungus and the host. Dispersion is another important virulence mechanism employed by *C. albicans*. Once the fungus has formed a biofilm, it can undergo a process called dispersion, in which individual fungal cells detach from the biofilm and disseminate to other sites within the host. This ability to disperse allows *C. albicans* to spread and establish infection in different areas, contributing to its pathogenicity. Proliferation, or the rapid growth and multiplication of *C. albicans cells*, is a key aspect of its virulence. The ability of the fungus to rapidly replicate within the host contributes to the severity and progression of the infection. By proliferating, *C. albicans* can overwhelm the host's immune defenses and further damage host tissues.

Damage to the host is another consequence of *C. albicans* virulence. The fungus can cause tissue destruction through the secretion of various enzymes, such as proteases and phospholipases, which degrade host proteins and lipids, respectively. This destruction of host tissues contributes to the pathogenicity of

*C. albicans* and aids in its invasion and spread within the host. Apart from the mechanisms mentioned above, there are specific genes associated with *C. albicans* virulence that have been extensively studied. This study focused on target genes involved in biofilm formation, namely HWP1, EFG1, and ALS3. HWP1 encodes a hyphal cell wall protein that is essential for biofilm formation, promoting the adhesion of *C. albicans* to surfaces. EFG1 is a transcription factor that regulates the switch between yeast and hyphal morphologies, and its expression is crucial for biofilm formation. ALS3 encodes an adhesin protein that plays a significant role in biofilm formation and adherence to host tissues. Additionally, the fourth virulence gene, ACT1, is associated with oxidative stress. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the host's ability to detoxify them. ACT1, which encodes an actin protein, has been implicated in the response of *C. albicans* to oxidative stress conditions. It is involved in the regulation of antioxidant defenses and the maintenance of redox homeostasis in the fungus.

In the oral cavity, *Streptococcus mutans,* another microorganism typically considered commensal, is known for its significant role in the development of dental caries. Both *C. albicans* and *S. mutans* are part of the oral microbiome, which consists of genetically unrelated microorganisms forming biofilms on dental surfaces. However, the specific relationship between *C. albicans* and *S. mutans* remains unclear.

In mixed species biofilms, co-inhabiting microbes can show gene expression changes that can affect their physiology and pathogenicity (Peters et

al., 2012). Moreover, cell-cell communication in biofilms depends on quorum sensing. Quorum sensing genes regulate the intercellular communication via cell density-dependent manner. (Albuquerque et al., 2012). As they can regulate host immunity and damage host cells, quorum sensing molecules are regarded as virulence factors (Winzer et al., 2001). CHK1 and PBS2 are genes in *C. albicans* that were found to be linked to quorum sensing and were tested in this study.

The expression of virulence and quorum sensing in *C. albicans* in mixed biofilms with *S. mutans* has not been adequately described. In this study, the effect of *S. mutan*s on *C. albicans* growth, virulence and quorum sensing expression was studied in a mixed-species biofilm populated by both organisms.

**Hypothesis**

The hypothesis made for this study was that, in a mixed-species biofilm inhabited by both *C. albicans* and *S. mutans*, the interactions between the two organisms can affect the growth of *C. albicans* as well as its expression of virulence and quorum sensing.

**Objectives**

The objectives of this study were:

1）To build individual *C. albicans* biofilms, and mixed-species biofilms co-inhabited by both *C. albicans* and *S. mutans*.

2）To compare the growth of *C. albicans* in single-species biofilms with the growth of *C. albicans* in mixed-species biofilms populated by both *C. albicans* and *S. mutans*.

3）To compare the expression of selected virulence and quorum sensing genes by *C. albicans* in single species *C. albicans* biofilms and mixed- species biofilms populated by both *C. albicans* and *S. mutans.*

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Candida albicans*

*Candida albicans*, a commensal organism, is commonly found in various locations within the human body, including the oral cavity, gastrointestinal tract, and genitourinary tract. *Candida* infection in the oral cavity, known as thrush, can be found among infants and elderly individuals. While candidiasis is not uncommon in clinical practice, it requires special attention, particularly in patients with compromised immune systems, as they are at a higher risk of developing invasive infections. Furthermore, factors such as radiotherapy, diabetes, and the use of steroids are associated with an increased susceptibility to *Candida* infections in patients. The potential consequences of these invasive infections can result in significant mortality rates, particularly in the intensive care units (Dimopoulos et al., 2008).

The transformation of *C. albicans* from a normal organism to a pathogen is the key to pathogenesis. *C. albicans* has different cell morphology including yeast cells, pseudohyphae and hyphae, and transformation among them depend on the condition of growth. The morphological transition is triggered by environmental signals such as changes in pH, oxygen or carbon dioxide concentrations, environmental temperature, availability of nutrients and the presence of serum in the human body (Brown et al. 2007). This capacity of change enables *C. albicans* to adapt rapidly and efficiently to different

environmental conditions. Due to this transformation between yeast cell and hyphae (pseudohyphae), the process of from commensal to pathogen occurs.

### 2.2 From commensal to pathogen

The role of *C. albicans* can change from being a commensal to becoming a pathogen due to alteration in host conditions and living environment. For instance, approximately 75% of women in their reproductive age will encounter at least one occurrence of vulvovaginal candidiasis (VVC). Furthermore, up to 9% of women in this group are affected by recurrent VVC, which is related to high estrogen level, contraceptives, or diabetes (Yano et al. 2019). Though *C. albicans* is found commonly as a commensal microorganism which can cause diseases in humans who are immunosuppressed, in particularly those with advanced diabetes mellitus or HIV infection, in whom infections are often severe or fatal. Infections occur when the microbiota at colonized sites is perturbed (d'Enfert et al., 2021). Predisposing factors of oral *C. albicans* infection include oral infections by other microbes, nutritional deficiencies, wearing of dentures, smoking, salivary hypofunction and local dysbiosis. *C. albicans* septicemia is often fatal and invasive fungal infections are a major cause of death among hospitalized patients (Brown et al., 2012). Candidiasis is difficult to diagnosis in the early stage and is also particularly challenging to treat because of resistance to drugs (Brown et al., 2012).

### 2.3 Virulence of *Candida albicans* and virulence genes

Microbial virulence refers to the capacity of a microorganism to invade a host and cause disease. It encompasses various abilities that enable the pathogen

to enter the host, circumvent host defense mechanisms, proliferate within the host environment, and inflict damage to host tissues (Leitão et al., 2020). *Candida albicans*, for instance, possesses several virulence factors such as adhesins, biofilm formation, secreted hydrolytic enzymes, phenotypic switching, immune invasion, and resistance to antifungal drugs (Calderone et al., 2001).

The virulence of *C. albicans* manifests through cellular polymorphism, the formation of biofilms on surfaces, penetration and growth within host cells, and the ability to withstand various stresses in both external and intracellular environments. The most important aspect of virulence could be the formation and initiation of biofilms, as they can facilitate the progression of other virulence factors. Surface adhesins facilitate the attachment of the fungus to host cells, while fungal enzymes aid in nutrient acquisition from the surrounding environment. Furthermore, the presence of various antioxidants provides protection against oxidative stress, including the respiratory burst (Chakravarti et al., 2017; Wysong et al., 1998). Virulence factors of *C. albicans* are encoded by its DNA, and genetic variations can occur, particularly in response to antifungal treatments, leading to alterations in bioactivity through molecular pathways (Bhattacharya et al., 2020).

Both the yeast and hyphal forms of *C. albicans* are essential and crucial for the complete expression of virulence (Noble et al., 2017). Studies suggest that many virulence-associated proteins are regulated by common genes, such as the Efg1p gene, which also controls cell morphology and biofilm formation (Kumamoto et al., 2005; Naglik et al., 2003; Schröppel et al., 2000; Lassak et

al., 2011). EFG1 is a transcription factor that regulates the transition from yeast to hyphal form, while HWP1 is involved in hyphal adhesion and biofilm formation.

Hwp1 is a cell wall protein that is localized to the outer layer of the fungal cell wall. It contains multiple adhesive domains that allow it to bind to various host components, such as extracellular matrix proteins, epithelial cells, and endothelial cells. By binding to these host surfaces, Hwp1 promotes the attachment of *Candida albicans* to the host tissues, facilitating its colonization and subsequent infection. In addition to its adhesive properties, HWP1 is also involved in biofilm formation, which is a complex community of fungal cells encased in a matrix. Biofilms provide increased resistance to antifungal agents and host immune defenses, making them a significant factor in *Candida albicans* infections. HWP1 contributes to biofilm formation by promoting cell-cell adhesion and the development of the extracellular matrix. Adhesins are cell surface proteins that facilitate the attachment of *C. albicans* to host tissues, enabling colonization and invasion. When the HWP1 gene is knocked out, resulting in a null mutant, the fungus remains viable but shows a significant reduction in its ability to form hyphae (Sharkey et al., 1999). Interestingly, although the function of this gene is correlated with virulence, overexpression of HWP1 has been found to have a negative impact on the aggressiveness of the *C. albicans* phenotype. This hyperexpression may be part of the fungus's response to changes in the host environment encountered during hyphae formation (Maras et al., 2021).

ALS3 (Agglutinin-like sequence 3) is a major adhesin gene that mediates binding to host cells and extracellular matrix components. ALS3 is also involved in biofilm formation and is crucial for the establishment of C. albicans infections. *C. albicans* encounters oxidative stress during host interactions due to the production of reactive oxygen species (ROS) by immune cells. ACT1 (Actin 1) is a virulence gene associated with oxidative stress response in *C. albicans*. It is involved in maintaining cellular integrity and protecting against ROS-induced damage. Actin cytoskeleton rearrangements mediated by ACT1 are essential for the pathogen's ability to invade host tissues and escape phagocytosis.

There are also other virulence genes related to virulence. The Secreted Aspartyl Protease (SAP) gene family encodes a group of virulence factors in *C. albicans*. SAP proteins contribute to the colonization and invasion of host tissues by degrading host proteins and interfering with immune responses. They also promote biofilm formation and modulate the host immune system by affecting cytokine production and immune cell function. Heat shock proteins (HSPs) are stress-responsive proteins that play a crucial role in protecting cells against various environmental stresses, including thermal stress. In *C. albicans*, HSP90 is an important virulence gene involved in morphogenesis, biofilm formation, and resistance to antifungal drugs. HSP90 aids in stabilizing and activating numerous client proteins required for fungal adaptation and survival within the host environment. The High Osmolarity Glycerol 1 (HOG1) pathway is a conserved signaling cascade involved in stress response and virulence in *C. albicans*. Activation of HOG1 promotes adaptation to osmotic stress, oxidative stress, and other environmental challenges encountered during infection. HOG1

regulates various aspects of *C. albicans* virulence, including biofilm formation, filamentation, and resistance to antifungal agents.

### 2.4 Quorum sensing and genes

Quorum sensing is a communicative activity among microbe cells linked to cell density. Individual microbe cell produces signaling molecules in low concentration but as the cell population increases, the signaling molecules produced will attain a level that is high enough to induce changes in gene expression. *Candida albicans,* one of the fungi, has been extensively studied in the context of quorum sensing for several decades. Farnesol is one of the quorum sensing molecules produced by *C. albicans*. It was showed that farnesol can inhibit biofilms formation of *C. albicans*. The second quorum sensing molecule is a derivative of phenethyl alcohol called tyrosol. Like farnesol, tyrosol has also been shown to affect the morphological transformation and biofilm formation of *C. albicans*. In in vitro experiments, both quorum sensing molecules exerted a greater (>80%) inhibition on the developing biofilm than on a pre-formed biofilm (<40%). However, unlike farnesol which inhibited the transformation of yeast cells to hyphae, tyrosol promoted hyphae development at the first 1-6 hours of biofilm formation (Sebaa et al. 2019). Furthermore, unlike farnesol whose biofilm inhibition was not affected in the absence of oxygen, tyrosol exerted much less inhibition on biofilm development when *C. albicans* was in anaerobic cultures, compared to the inhibition observed in aerobic cultures (Alem et al., 2006).

Within the biofilm, cells exchange signals by quorum sensing (Li et al.

2022). This concept was first demonstrated in 1970 when it was found that a luminescent marine bacterium only produced light in densely populated cultures (Nealson et al. 1970). Compared with other fungal species, *C. albicans* has been well studied for quorum sensing (Tong et al., 2017; Barriuso et al., 2018). The first fungal quorum sensing system was reported by Hornby et al. (Hornby et al. 2001). Shirtliff et al. (2009) described farnesol, an alcohol now known for its multiple effects on gene functions including the down regulation of biofilm development, hyphae production, glycolysis and protein synthesis, as well as the upregulation of apoptosis, protein folding, and protection against stress from reactive oxygen species (Shirtliff et al., 2009b; McIlwain et al. 2013). This indicated a gene-regulated activity that is induced in response to an increase in cell density and is most likely beneficial to commensal microbe and their hosts (Jugder et al., 2022).

CHK1 has been identified as the gene responsible for quorum sensing in *C. albicans,* which can be upregulated by farnesol (Kruppa et al. 2009). However, recent findings have revealed that CHK1 possesses additional functions beyond their involvement in quorum sensing (Zhou et al. 2021). According to another research, CHK1 plays a role in hyphal formation and stress response, showcasing its multifaceted nature (Cheetham et al. 2007). HOG1 is another gene that was upregulated by farnesol. PBS2 encodes MAP kinase and plays an essential role in HOG1 pathway for oxidative stress (Arana et al. 2005). Hence, CHK1, PBS2 and HOG1 are important genes linked to quorum sensing and virulence.

## 2.5 Interaction between *Candida albicans* and *streptococcus mutans*

The communication between *C. albicans* and *S. mutans* illustrates distinct ways of interaction among microbes from different microbial kingdoms. There are conflicting reports in the literature regarding these interactions, which may be synergistic or antagonistic, as seen in the varied outcomes of contact between *C. albicans* and *S. mutans, a* predominant bacterial species in dental plaques (Sztajer et al., 2014). In biofilms housing both organisms, it has been observed that the induction of quorum sensing in *S. mutans* resulted in an increase in the *C. albicans* population, and *C. albicans* promoted the growth of *S. mutans* with the quorum sensing molecule farnesol (Kim et al., 2017). In contrast, it was reported that *S. mutans* inhibited candidiasis in a mouse model (dos Santos et al., 2020). Xu et al also described growth synergism between *C. albicans* and another oral commensal *S. oralis*, which led to more serious *candida* infections in the buccal cavity and spread of infection to deep body organs (Xu et al. 2014). Among the published studies, most were focused on the influence of *C. albicans* on *S. mutans* with respect to gene expression while few looked at the effect of *S. mutans* on *C. albicans*, underscoring the need for investigations in this latter scenario.

## 2.6 Quorum sensing and virulence of *C. albicans*

Quorum sensing of *C. albicans* is communication based on the cell density. It involves the production and detection of signaling molecules, which enable the cells to sense their own population density. Quorum sensing in *C. albicans* has been linked to its virulence, which refers to the ability of the organism to

cause disease. In *C. albicans*, quorum sensing regulates the expression of various virulence factors that contribute to its pathogenicity. These virulence factors include adhesion molecules, secreted hydrolytic enzymes, biofilm formation, and the transition between different morphological forms of the fungus (Kruppa et al.2009). The ability of *C. albicans* to switch between yeast and filamentous forms is particularly important for its pathogenicity, as the filamentous form is more invasive and better able to penetrate tissues. Interestingly, other factors, such as temperature, pH, and the presence of host-related molecules, can also influence the virulence of *C. albicans* and its response to quorum sensing. For example, the transition to filamentous forms can be triggered by physiological conditions encountered in the host, such as elevated temperature and pH value. Quorum sensing allows the fungus to coordinate the expression of virulence factors in response to changes in cell density and environmental cues, ultimately contributing to its ability to cause infections in humans.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Microbial strains

### 3.1.1 *C. albicans*

*C. albicans* ATCC 10231 strain archived at -80℃ in TSB (BD, USA) containing 15% glycerol were revived on Sabouraud Dextrose agar (SDA) (OXOID, UK) plates incubated at 37°C overnight. The isolation source of *C. albicans* ATCC 10231 is taken from a man with bronchomycosis. Colony obtained on SDA were sub-cultured to obtain pure, viable *C. albicans* cells for the construction of single-species and mixed-species biofilms.

### 3.1.2 *S. mutans*

*S. mutans* ATCC 25175 strain archived at -80℃ in Brain Heart Infusion (BHI) (Himedia, India) broth containing 15% glycerol were revived on BHI agar (Himedia, India) plates incubated at 37°C for 48 hours. The isolation source of *S. mutans* ATCC 25175 is taken from carious dentine. Colony obtained were sub-cultured to obtain pure, viable cells for the construction of mixed-species biofilms.

### 3.2 Biofilm construction

3.2.1 Selection of liquid culture medium for growing single-species and mixed-species biofilms

To ensure a correct comparison in gene expression assays, the single and mixed- species biofilms of *C. albicans* needed to be cultivated using the same growth medium. To choose a suitable medium, one colony each of *C. albicans* and *S. mutans* was cultured separately in different tubes of SDB and incubated with shaking for 18 Hours at 37℃ and 250 rpm. Although the SDB culture of *C. albicans* yielded heavy growth, *S. mutans* did not grow in the same broth medium. This indicated that SDB was not a suitable medium for the formation of a mixed *C. albicans* and *S mutans* biofilm.

Following further experimentation, it was found that, BHI broth and BHI broth with 5% Fetal bovine Serum (FBS) supported both the growth of *C. albicans* and *S. mutans* single-species and mixed-species biofilms. However, the biofilms developed were still unsatisfactory. By further trial and error, a combination of BHI broth and SDB (1:1) was eventually found to be able to support good growth of both *C. albicans* and *S. mutans* (Figure 1, 2). Hence, this broth combination was used for growing all single-species and mixed-species biofilms of *C. albicans* and *S. mutans* in this study.

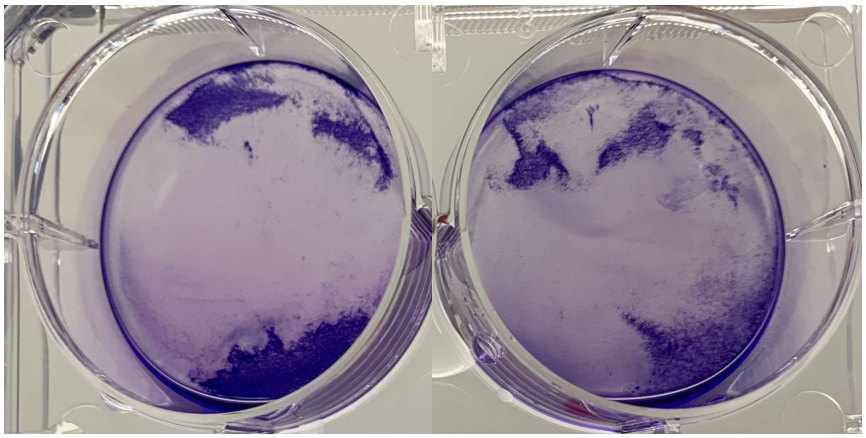


Figure 1. Poor growth of *C. albicans* (left) and *C. albicans* +*S. mutans* (right) biofilms in BHI broth.

Figure 2. Abundant growth of *C. albicans* (left) and *C. albicans* +*S. mutans* (right) biofilms developed in SDB: BHI broth (1:1)

Single-species *C. albicans* biofilms and mixed-species biofilms of *C. albicans* and *S. mutans* were developed in six-well treated tissue culture plates. After incubation, washing and Gram staining, biofilms were observed to be adhering to the bottom of the wells (Figure 3).

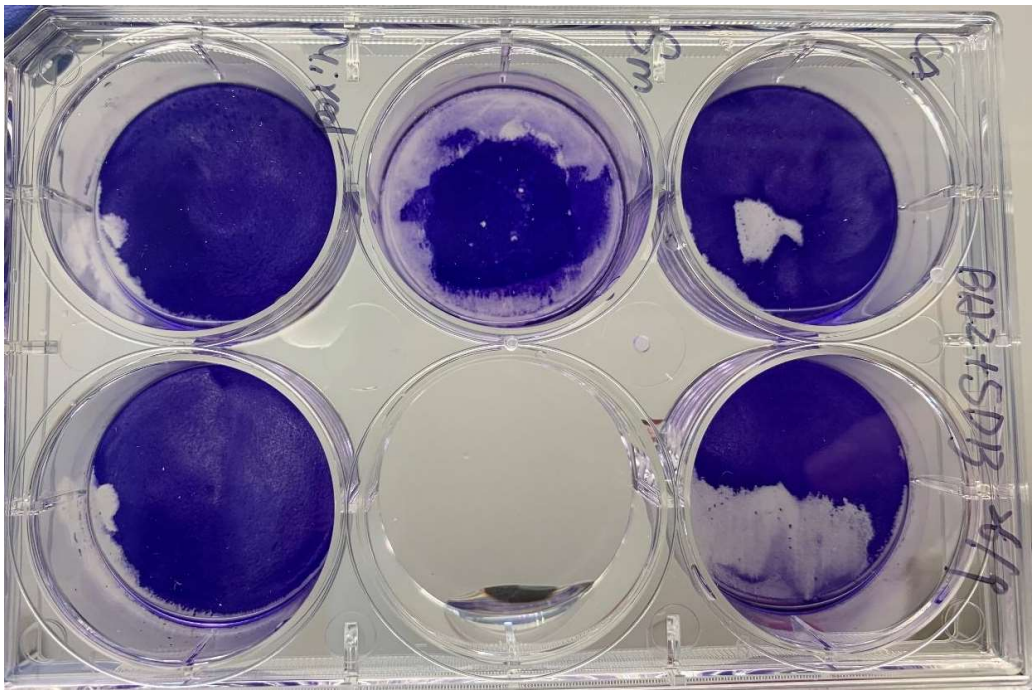


Figure 3. Stained biofilms adhering to the bottom of the wells in single-species and mixed-species biofilms.

Under the inverted microscope, the biofilms showed characteristic structures of *C. albicans* and *S. mutans* (Figures 4 and 5).

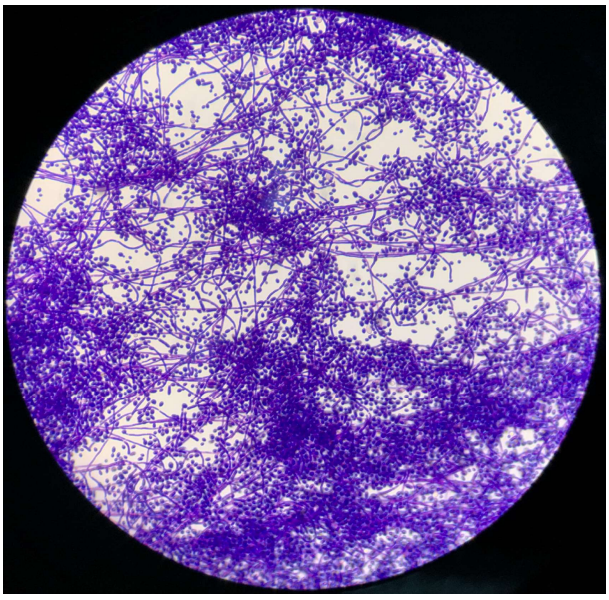


Figure 4. Gram-stained hyphae and yeast cells of a single-species *C. albicans* biofilm observed under the inverted microscope at 400 x magnification.

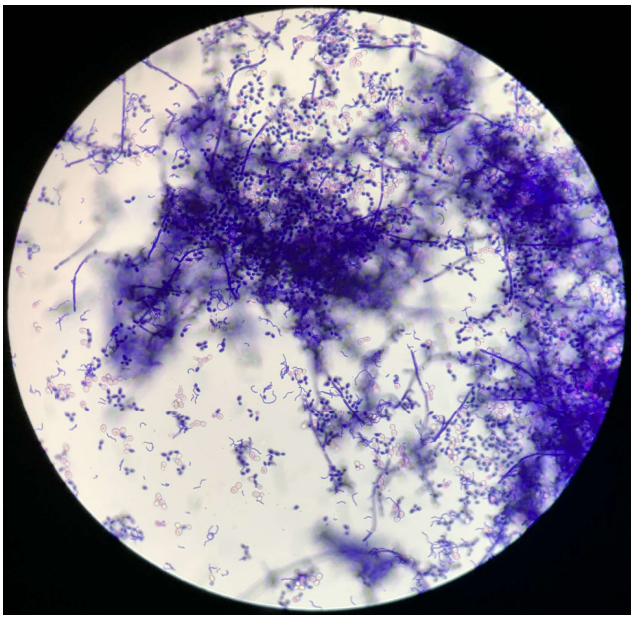


Figure 5. Gram-stained hyphae and yeast cells of *C. albicans* and short chains of *S. mutans* cocci in a mixed-species biofilm observed under the inverted microscope at 400 x magnification.

3.2.2 Measurement of cell density and determination of viable counts

3.2.2.1. Determining the viable count of a 5 McFarland (McF) suspension of *C. albicans*

A colony of *C. albicans* ATCC 10231 was cultured in a 1:1 mixture of BHI broth and SDB for 18h at 37ºC and 250 rpm in a shaking incubator. At the end of incubation, the cell density was measured with a McF densitometer and adjusted with fresh broth to a 5 McF suspension. This suspension was serially diluted from $10^1$ to $10^6$ times in 6 tubes. From each tube, triplicate 10µl suspensions were pipetted onto a BHI agar plate to obtain colony forming units (CFU) using the Miles and Misra method (Hedges AJ, 1978). After overnight incubation at 37ºC, the viable count (CFU/ml) of *C. albicans* was determined by the formula below. For the 5 McF suspension, the viable count was calculated to be 1.5×10^7/ml.

CFU/ml= (Number of colonies × dilution factor) / volume of culture plate (ml).

The Miles and Misra protocol for CFU (colony-forming unit) quantification was utilized in this study, and the specific details can be found in Appendices X. To determine the CFU, counts were conducted for each triplicate and subsequently averaged. The average number of colonies were used as "number of colonies" in the formula.

3.2.2.2 Determining the viable count of a 3.5 McF suspension of *S. mutans*

A colony of *S. mutans* ATCC 25175 was cultured in BHI:SDB (1:1) for 18h at 37ºC at 250 rpm in a shaking incubator. At the end of incubation, the cell density was measured with a McF densitometer and then adjusted with fresh broth to McF standard 3.5 accurately. The suspension was then serially diluted from $10^1$ to $10^6$ times in 6 tubes. From each tube, triplicate 10µl suspensions were pipetted onto a BHI agar plate to obtain CFU. The CFU were obtained through method of Miles and Misra which was described above. After overnight incubation at 37ºC with 5% $CO_2$, the CFU/ml of *S. mutans* at McF 3.5 was determined to be 1.5×10^8/ml.


Figure 6. Densitometer for measuring cell density.

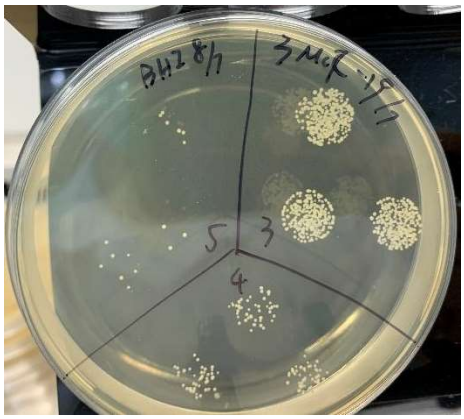



Figure 7. Miles and Misra method for the determination of CFU/ml

3.2.3 Construction of pure *C. albicans* biofilms and determination of viable counts

From a 5 McF suspension of *C. albicans* cells in BHI:SDB (1:1), 4ml aliquots were pipetted into each well of a 6-well untreated tissue culture plate (Orange Scientific, USA) for incubation at 37℃ for 90 minutes to allow planktonic cells to adhere to the bottom surface of the well. After the 90 min incubation, the broth was carefully aspirated and the wells were washed 3 times with PBS, following which, 4ml of fresh broth was added to each well for another 24 hours of incubation at 37℃ to allow for biofilm formation from sessile cells.

At the end of the 24 hours incubation, each biofilm was washed twice with Phosphate-buffered saline (PBS). After the second wash, biofilm cells from one well were dislodged into 1ml PBS, using a cell scraper, and the cell suspension was aspirated into a test tube for the determination of viable count using the Miles and Misra assay as described in section 3.2.2.1. Biofilms in the remaining 5 wells were kept for RNA extraction which is described in section 3.6. The CFU/ml of *C. albicans* biofilms was calculated using the formula described in section 3.2.2.1.

### 3.2.4 Construction of mixed-species biofilms with different ratios and determination of viable counts

*C. albicans* was grown in BHI:SDB (1:1) and adjusted to 5 McF after incubation. Four ml of 5 McF *C. albicans* suspension was used to make single biofilms in each well of a 6-well plate. Similarly, *S mutans* was grown in BHI:SDB (1:1) and adjusted to 3.5 McF after incubation. Equal volumes of 5 McF and *C. albicans* and 3.5 McF *S. mutans* were mixed and 4 ml of the mixture were pipetted into each well of a 6-well untreated tissue culture plate for the development of mixed-species biofilms with the ratio of 1:10 (*C. albicans*: *S. mutans*).

Two sets biofilms were produced. Four ml of 5 McF *C. albicans* suspension was used to make single biofilms in each well of a 6-well plate (Orange Scientific, USA). Two ml of 5 McF *C. albicans* suspension and 2ml of 3.5 McF of *S. mutans* suspension were used to make mixed-species biofilms of

1:10 cell number ratio in each well of a 6-well plate. The 3.5 McF suspension of *S. mutans* was diluted three-fold, and 2ml of the diluted suspension was added with 2ml of 5 McF *C. albicans* suspension to make mixed-species biofilms of 1:3 ratio in each well of a 6-well plate.

The plates containing various suspensions with different ratios were incubated at 37℃ for 90 minutes to allow cell attachment to the bottom of the wells. Afterward, they were washed three times with PBS. Following the final wash, 4 ml of fresh broth was added to each well, and the plates were further incubated at 37℃ for 24 hours to promote biofilm development.

At the end of incubation, the biofilms in every well were washed twice with PBS. After the second wash, biofilm cells from each well were dislodged into 1ml PBS and the suspension was aspirated into a tube. Two sets biofilms were produced:

Dilutions of the suspension with the ratio of 1:10 from one well was made from $10^1$ to $10^6$ times in 6 tubes, while the suspensions from the other 5 wells were kept for RNA extraction. Triplicate 10µl aliquots from each of the 6 tubes were inoculated onto a) BHI agar for the growth of *S. mutans* and *C. albicans*, and b) CHROMagar (CHROMagar *candida*, USA, Difco) for the isolation of *C. albicans* only, as the chloramphenicol in CHROMagar inhibits the growth of *S. mutans*. All agar plates were incubated overnight at 37ºC with 5% $CO_2$ after which, the number of colonies grown on the various agar plates were counted. The colony count on BHI agar was taken to be the total CFU count for both *C.*

*albicans* and *S. mutans*. The CFU count of *C. albicans* was obtained from the CHROMagar while the CFU count of *S. mutans* was obtained by subtracting the count on CHROMagar from that on the BHI agar. The CFU/ml of *C. albicans* and *S. mutans* were then calculated.

Dilution of the suspension with the ration of 1:3 from wells were made from $10^1$ to $10^6$ times in 6 tubes. Triplicate 10µl aliquots from each of the 6 tubes were inoculated onto a) BHI agar for the growth of *S. mutans* and *C. albicans,* and b) CHROMagar (CHROMagar candida, USA, Difco) for the isolation of *C. albicans* only, as the chloramphenicol in CHROMagar inhibits the growth of *S. mutans*. All agar plates were incubated overnight at 37℃ with 5% CO2 after which, the number of colonies grown on the various agar plates were counted. The colony count on BHI agar was taken to be the total CFU count for both *C. albicans* and *S. mutans*. The CFU count of *C. albicans* was obtained from the CHROMagar while the CFU count of *S. mutans* was obtained by subtracting the count on CHROMagar from that on the BHI agar. The CFU/ml of *C. albicans* and *S. mutans* were then calculated.

The *C. albicans* CFU/ml counts in single species and mixed species biofilms with different ratios are shown in Table1.

Table 1. *C. albicans* and *S. mutans* viable counts (CFU/ml) in single and mixed-species biofilms

| Biofilms | CFU-1 | CFU-2 | CFU-3 | CFU-average |
|---|---|---|---|---|
| *C. albicans*-Single species | 2.3×10^7 | 2.0×10^7 | 3.3×10^7 | 2.5×10^7 |
| *C. albicans*-Mixed species at 1:3 | 5.0×10^7 | 8.6×10^7 | 3.6×10^7 | 5.7×10^7 |
| *S. mutans*-Mixed species at 1:3 | 2.3×10^7 | 2.0×10^7 | 2.0×10^7 | 2.1×10^7 |
| *C. albicans*-Single species | 3.2×10^7 | 2.9×10^7 | 3.6×10^7 | 3.2×10^7 |
| C. albicans-Mixed species at 1:10 | 3.2×10^7 | 2.9×10^7 | 4.3×10^7 | 3.4×10^7 |
| S. mutans-Mixed species at 1:10 | 3.1×10^7 | 3.1×10^7 | 3.3×10^7 | 3.2×10^7 |

3.2.5 Viable counts of suspensions of *C. albicans* and *S. mutans* used for growth of biofilms.

As described in previous sections, *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175 were used to make suspensions in BHI:SDB (1:1) and adjusted to different McF standards. For each McF standard, the corresponding CFU/ml viable count was determined with the Miles and Misra method. Suspensions of *C. albicans* and *S. mutans* with different CFU/ml were then mixed to achieve different *C. albicans*: *S. mutans* ratios as shown in Table 2.

Table 2. Viable counts of suspension of *C. albicans* and *S. mutans* at different concentrations.

| *C. albicans* | | *S. mutans* | | Ratio of *C. albicans: S. mutans* |
|---|---|---|---|---|
| McF | CFU/ml | McF | CFU/ml | |
| 5.0 | 1.5×10^7 | 3.5 | 1.5×10^8 | 1/10 |
| 5.0 | 1.5×10^7 | 1.0 | 5.0×10^7 | 1/3 |

3.2.6 *C. albicans* viable counts in a single-species biofilm developed with inoculant of different concentrations.

For the formation of a single-species biofilm, the inoculum used 4ml of a 5 McF suspension of *C. albicans*. For the formation of a mixed-species biofilm, the inoculum was 2ml of a 5 McF suspension of *C. albicans* plus 2ml of a 3.5 McF suspension of *S. mutans.* Hence the *C. albicans* in the inoculum for the mixed-species biofilm was only half that used in the single-species biofilm. Nonetheless, the results (Table 3) showed no significant difference (p=0.89) in *C. albicans* CFU/ml in biofilms developed from either a 5McF standard suspension or a 5 McF standard suspension diluted 1:2.

Table 3. *C. albicans* viable counts (CFU/ml) in biofilms developed from a 5 McF suspension and a 1:2 diluted 5 McF suspension of *C. albicans*

| *C. albicans* Suspension | CFU/ml | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | Average |
| 5.0 McFarland inoculum | 5.3×10^7 | 3.6×10^7 | 3.0×10^7 | 3.96×10^7 |
| 5.0 McFarland diluted 1:2 | 4.3×10^7 | 2.6×10^7 | 4.6×10^7 | 3.83×10^7 |
| Unpaired t-test p-value | | | | 0.8927 |

### 3.3 RNA extraction and purification

For RNA extraction, single-species, and mixed-species (1:10 ratio) biofilms developed as described in sections 3.2.3. to 3.2.5 were harvested and treated with DNase on a column employing the Qiagen RNeasy Mini kit (Qiagen, Germany). The RNA collected was then subjected to a second DNase I treatment and purified via the Qiagen RNeasy MinElute cleanup kit (Qiagen, Germany). These RNA extraction and purification procedures were performed using an

optimized protocol (Nailis et al. 2006). RNA quantification was carried out using the BioSpec-nano spectrophotometer (NanoDrop One Themo Scientific, US). Triplicate RNA samples were taken from single and mixed-species biofilms. More details of RNA extraction and purification are provided in Appendices U and V.

### 3.4 Reverse transcription of RNA

From each of the RNA samples, a standard amount of RNA was used for conversion to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO); the protocol provided by TOYOBO company, Japan (Farkas et al., 2009) was strictly followed. More details of the process of reverse transcription are provided in Appendices W.

### 3.5 RT-qPCR

#### 3.5.1 Target genes

Two quorum sensing genes and 4 virulence genes of *C. albicans* were selected for Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) (Table 4). The sequence of each target gene was confirmed with NCBI-GENE.

Table 4. *C. albicans* virulence genes and their functions

| Gene | Function |
|---|---|
| CHK1 | Quorum sensing, hyphal formation, biofilm formation (Zhou et al. 2021) |
| PBS2 | Quorum sensing, cell-wall construction, oxidative and osmotic stress (Arana et al. 2005) |
| HWP1 | Hyphal wall protein (adherence to epithelial cells) (Maras et al., 2021) |
| EFG1 | Yeast-to-hyphae transition (Kumamoto et al., 2005） |
| ALS3 | Adherence (to vein endothelial cells and buccal epithelial cells) Liu et al.2011） |
| CAT1 | Oxidative stress (encoding catalase) (Chakravarti et al, 2017) |

3.5.2 Primers

The primers used for gene amplification (Table 5) were designed with the primer designer tool in the NCBI website primer system (https://www.ncbi.nlm.nih.gov/gene). The best primers were chosen based on the parameter of self-complementarity which should be less than 5. Though *S. mutans* and *C. albicans* are from different taxonomic kingdoms, it is still necessary to confirm that all the primers to be used would not amplify any nucleotide sequence in *S. mutans*. Two methods were used to test this. Firstly, PCR was used to amplify *C. albicans* target genes in *S. mutans;* the results were negative (Figure 8). Secondly, The NCBI website was used to check whether the primers for the four virulence genes, HWP1, EFG1, ALS3 and CAT1, share similar nucleotide sequences in the genome of *S. mutans*.

The check did not reveal any matching sequence in *S. mutans* for all the primers through the method below (more details in Appendices Y). The primers were designed using the NCBI website primer system, Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). This same system was used

to confirm that the primers would not bind to any sequences in the *S. mutans* genome.

Primers for the four virulence genes (HWP1, EFG1, ALS3 and CAT1) were designed through NCBI website of National Library of Medicine of the U.S.

Table 5: Primer sequences for the PCR amplification of house-keeping genes (ACT1, RPP2B, PMA1), quorum sensing genes (CHK1, PBS2), and virulence genes (HWP1, EFG1, ALS3, CAT1)

| Gene | Primers | Sequence | Product size(bp) |
|---|---|---|---|
| ACT1 | forward | TGCTGAACGTATGCAAAAGG | 20 |
| | reverse | TGAACAATGGATGGACCAGA | 20 |
| RPP2B | Forward | ATATCACCGCTTTATTGGAATCCG | 23 |
| | reverse | GATGGGACAGAAGCTAATTTGG | 22 |
| PMA1 | forward | CCAGAGCTCAAGGTCCATTCT | 21 |
| | reverse | AGTGTGTTGTTGTGGTTTTCTAC | 23 |
| CHK1 | forward | AGATGGTTCCGCAAGACGAG | 20 |
| | reverse | TGTGCCAATCCCTTCCTCAC | 20 |
| PBS2 | forward | TACGCCAGAAGCAGATAC | 18 |
| | reverse | TGAAGACCCAGACAGAAA | 18 |
| HWP1 | forward | CCGGAATCTAGTGCTGTCGT | 20 |
| | reverse | TTGGCAGATGGTTGCATGAG | 20 |
| EFG1 | forward | ACGAGTAACAACTACCAT | 18 |
| | reverse | TATCTGCTCTTCTGACAA | 18 |
| ALS3 | forward | TATTTGTCGGTTGCGACTGC | 20 |
| | reverse | TGGGGTTCCTGGTCCCTTAT | 20 |
| CAT1 | forward | GACTGCTTACATTCAAAC | 18 |
| | reverse | AACTTACCAAATCTTCTCA | 19 |

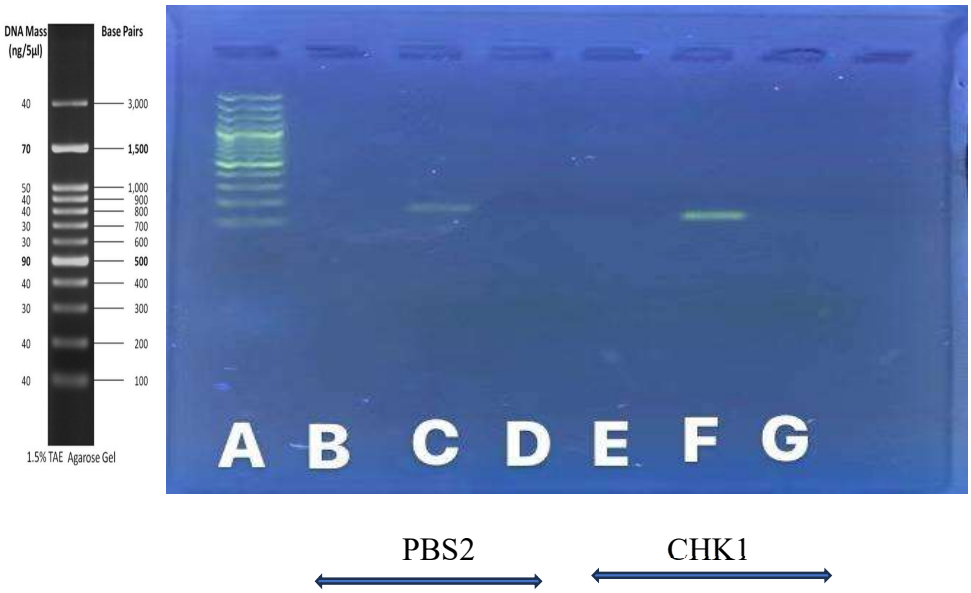



Figure 8. Gel electrophoresis picture for the PCR amplified quorum sensing genes CHK1 and PBS2, showing positive amplification in *C albicans* but not in *S mutans.*

A: 100 base-pair ladders
B: NTC with PBS2 primers,
C: *C. albicans* DNA with PBS2 primers,
D: *S. mutans* DNA with PBS2 primers,
E: NTC with CHK1 primers,
F: *C. albicans* DNA with CHK1 primers,
G: *S. mutans* DNA with CHK1 primers.

### 3.5.3 RNA concentrations from single-species and mixed-species biofilms

For the testing of gene expression, RNA was extracted from triplicate samples of single-species and mixed-species (1:10 ratio) biofilms. The concentration and purity of the extracted RNA are shown in Table 6.

Table 6: Quantity and quality of RNA extracted from single-species and mixed-species biofilms.

| Source of RNA | Concentration(ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|
| Single biofilms (1) | 337.5 | 2.23 | 2.73 |
| Single biofilms (2) | 91.0 | 2.15 | 2.51 |
| Single biofilms (3) | 260.5 | 2.20 | 2.72 |
| Mixed biofilms (1) | 284.0 | 2.23 | 2.89 |
| Mixed biofilms (2) | 304.6 | 2.22 | 2.53 |
| Mixed biofilms (3) | 128.8 | 2.23 | 2.75 |

3.5.4 Quantity and quality of post-purification RNA

RNA extracted from biofilms was further purified to remove any remaining contaminating DNA and extraction reagents. The results after further purification are shown in Table 7.

Table 7: Quantity and quality of extracted RNA after further purification

| Source of RNA | Concentration(ng/µl) | A260/A280 | A260/A230 |
|---|---|---|---|
| Single biofilms (1) | 223.9 | 2.15 | 2.12 |
| Single biofilms (2) | 83.3 | 1.87 | 1.16 |
| Single biofilms (3) | 175.2 | 2.00 | 1.49 |
| Mixed biofilms (1) | 138.9 | 2.17 | 2.31 |
| Mixed biofilms (2) | 162.4 | 2.16 | 2.42 |
| Mixed biofilms (3) | 36.9 | 2.12 | 0.33 |

## 3.6 Statistical analysis

3.6.1 CFU/ml of single species biofilms and mixed species biofilms

The viable counts (CFU/ml) of *C. albicans* of single-species biofilms and mixed-species biofilms prepared using different ratios of *C. albi*cans and *S. mutans* were calculated using the logarithmic function in Excel software. For the comparison of viable counts obtained for the three groups (single species, mixed species at 1:3 ratio and mixed species at 1:10 ratio), the One-Way-ANOVA analysis was used with the GraphPad software to look for any significant difference among the three groups compared. Following the detection of significant difference, the Tukey HSD test was used as a post-hoc test to determine which pair of groups was responsible for the significant difference.

Results of the One-Way ANOVA analysis to determine the significance of difference between the three groups of biofilms: single biofilms, mixed biofilms with a ratio of 1:3, and mixed biofilms with a ratio of 1:10 are shown in Table 8. As the p-value of the comparative analysis was significant with a p value of less than 0.05 (p=0.0342), the Tukey multiple comparison test was next performed. As shown in Table 9, the difference of CFU/ml between single biofilms and mixed biofilms with the ratio of 1:10 was not significant, neither was the difference between that of mixed biofilms with the ratio of 1:10 and 1:3 (the p values were 0.6932, and 0.1634 respectively). However, it was noted that difference in the CFU/ml counts between the single species and 1:3 mixed-species biofilms was statistically significant with a p-value of 0.0280.

Table 8. Results of the one-way ANOVA

| Ordinary one-way ANOVA, ANOVA results | |
|---|---|
| P value | **0.0342** |
| P value summary | * |
| Significant difference among means (P<0.05) | Yes |

Table 9. Results of Tukey's multiple comparison test

| Tukey's multiple comparisons test | Significant | Summary | Adjusted P Value |
|---|---|---|---|
| Single vs. Mixed 1:3 | Yes | * | **0.0280** |
| Single vs. Mixed 1:10 | No | ns | 0.6932 |
| Mixed 1:3. Mixed 1:10 | No | ns | 0.1634 |

3.6.2 RT-qPCR

RT-qPCR (35 cycles) was performed in 20ul tubes, each containing 1ul (about 740ng) cDNA of the test or the control sample. The PCR master mix (TOYOBO, Japan) and the Rotor-Gene Q Series Software (Qiagen, USA) were used. The reaction components of the amplification are listed in Table 10. Of

the 3 housekeeping genes ACT1, PMA1 and RPP2B (Nailis et al. 2006), ACT1 was selected for the RT-qPCR assay as it was identified by the RefFinder tool (DOI: 10.1007/s11103-012-9885-2), to be the most stably expressed (Figure 9).


Figure 9. Reference house-keeping genes for the RT-PCR. The RefFinder showed that ACT1 was the most stably expressed gene across the *C. albicans* samples from single and mixed biofilms.

Table 10. RT-qPCR reaction components

| Component | Volume (µl) |
|---|---|
| Master mix | 10.0 |
| Forward primer | 0.4 |
| Reverse primer | 0.4 |
| cDNA | 1.0 |
| $H_2O$ | 8.2 |
| Total | 20.0 |

The RT-qPCR amplification curves and the linear correlation co-efficacy were analyzed using the Rotor gene software (figure 10). For each target gene, 3 biological replicates were tested in 2 technical duplicates. The quantification values of target and reference genes from each sample were used to get the gene

expression ratio (expression in mixed- species biofilm divided by expression in single-species biofilm). A gene expression ratio >1 means higher expression in the mixed-species biofilm; a ratio <1 means lower expression in the mixed-species biofilm, and ratio=1 means no difference in expression between the single-species and mixed-species biofilms. The statistical significance of data was analyzed by the unpaired t-test method using Microsoft Excel software. A p-value of less than 0.05 was considered statistically significant.

For gene expression analysis using RT-qPCR, all primers designed for *C. albicans* genes underwent a thorough confirmation step to ensure their specificity to *C. albicans*. This confirmation was achieved through PCR assays conducted prior to their utilization in the RT-qPCR experiments. All samples for single-species and mixed-species biofilms were diluted to obtain the same RNA concentration before conversion to cDNA and gene amplification.

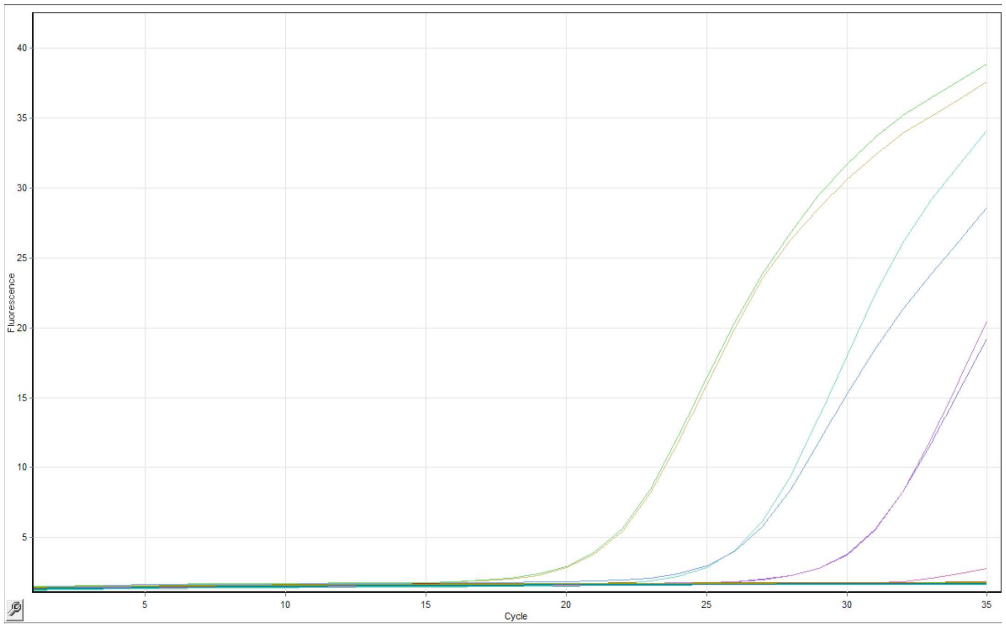


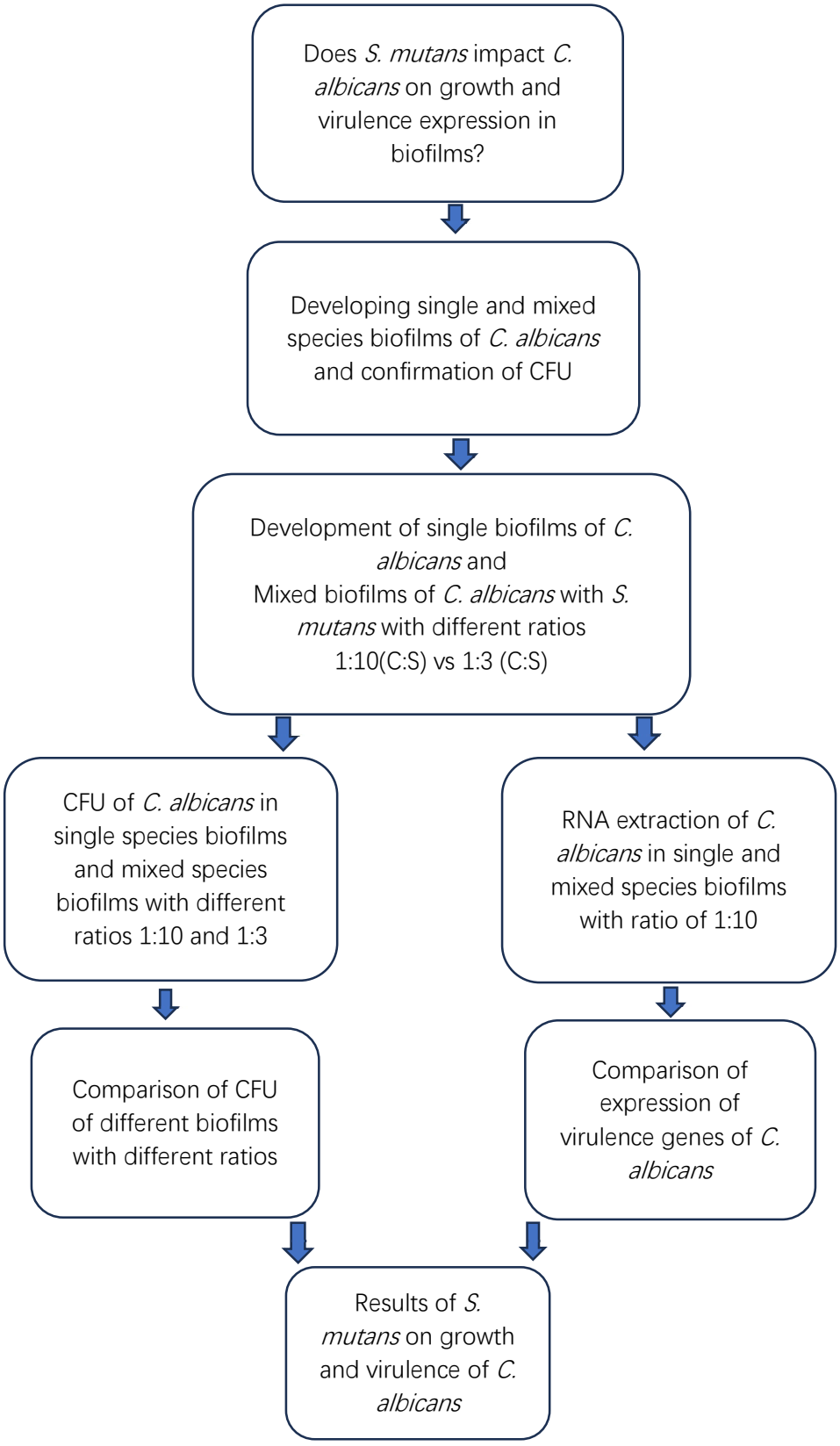

Figure 10. Amplified standard curves obtained from serial dilutions of cDNA of target gene HWP1.

The difference in gene expression between the single-species and mixed-species biofilms was expressed as a gene expression ratio obtained by dividing the gene expression (RT-qPCR quantification value) in a mixed-species biofilm over that in a single-species biofilm. The unpaired T-test was used to assess the significance of the difference calculated for each target gene (Tables A to I in Appendices L to T). The results showed no significant difference in gene expression between mixed-species and single-species biofilms, for all the genes examined. Although the expression ratio was greater than 1 for 4 of the genes, the p-values were all >0.05 (Table 11).

Table 11: Comparison of gene expression in single and mixed (1:10) species biofilms of *C. albicans*. QS genes: CHK1, PBS2. Virulence genes: HWP1, EFG1, ALS3, CAT1.Gene expression ratio>1 means higher expression in mixed-species biofilms, ratio <1 means lower expression and ratio=1 means no difference in expression.

| Gene | Expression ratio of gene (Mixed species biofilm /Mono species biofilm) | P value (Obtained with unpaired t-test) | Interpretation |
|---|---|---|---|
| CHK1 | 1.2596 | 0.4369 | Not significant |
| PBS2 | 1.3799 | 0.3969 | Not significant |
| HWP1 | 0.6610 | 0.5316 | Not significant |
| EFG1 | 1.1138 | 0.6964 | Not significant |
| ALS3 | 1.3565 | 0.2133 | Not significant |
| CAT1 | 0.8418 | 0.8292 | Not significant |

Flow Chat of this Study



# CHAPTER 4

# RESULTS

## 4.1 Results of biofilms

In this study, it was found that a cost-effective medium for cultivating mixed-species biofilms containing both *C. albicans* and *S. mutans* is a 1:1 ratio of mixed broth consisting of SDB and BHI broth. While previous papers have described various formulations, our studies have shown that a mixed broth consisting of SDB and BHI broth at a 1:1 ratio of supported satisfactory growth of both organisms in in mixed biofilms. This provides an inexpensive alternative that is suitable for the purpose of developing mixed-species biofilms of *C. albicans* and *S. mutans*.

## 4.2 Results of comparison of *C. albicans* viable counts in single and mixed-species biofilms

To investigate the impact of *S. mutans* on the growth of *C. albicans*, we conducted experiments using both single-species and mixed-species biofilms. These biofilms were developed under the same conditions, including identical media composition, *C. albicans* concentration and growth conditions. Our hypothesis proposed that the presence of *S. mutans* in mixed-species biofilms would affect the growth of *C. albicans* by altering the growth conditions. The graph (Figure 11) showed that in the mixed species biofilms, the *C. albicans* counts were higher than those in the single species biofilm. The results,

expressed as colony-forming units (CFU) counts, demonstrated significant differences between single-species and mixed-species biofilms when the ratio of *C. albicans* to *S mutans* was 1:3. Hence, it appears that that the growth of *C. albicans* was significantly influenced by the presence of *S. mutans* in mixed-species biofilms at a particular level of abundance of the latter. We conclude therefore that *S. mutans* promoted the growth of *C. albicans* under the experimental conditions used in this study.

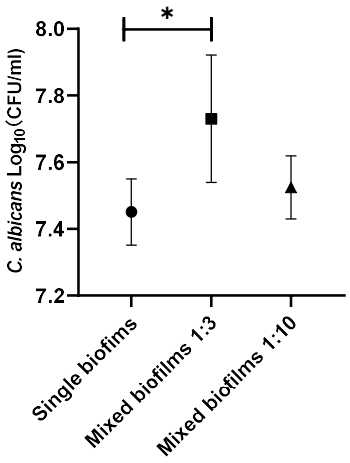



Figure 11. Comparison of *C. albicans* viable counts in single and mixed species biofilms. *Significant difference (p-value 0.0280)

### 4.3 Results of comparison of virulence and QS gene expression

With regards to the influence of *S mutans* on gene expression in *C albicans*, the hypothesis was that the presence of *S. mutans* in mixed-species biofilms would affect the expression of quorum sensing (QS) genes and virulence genes in *C. albicans.* However, the results of our studies supported the Null hypothesis that there is no significant difference in the level of expression of the *C albicans* target genes between single and mixed species cultures. Therefore, our study indicates that the presence of *S. mutans* in mixed-species biofilms did not have

a significant impact on the expression of the tested genes in *C. albicans*, suggesting that *S. mutans* did not significantly influence the virulence of C. albicans in this context (figure 12).

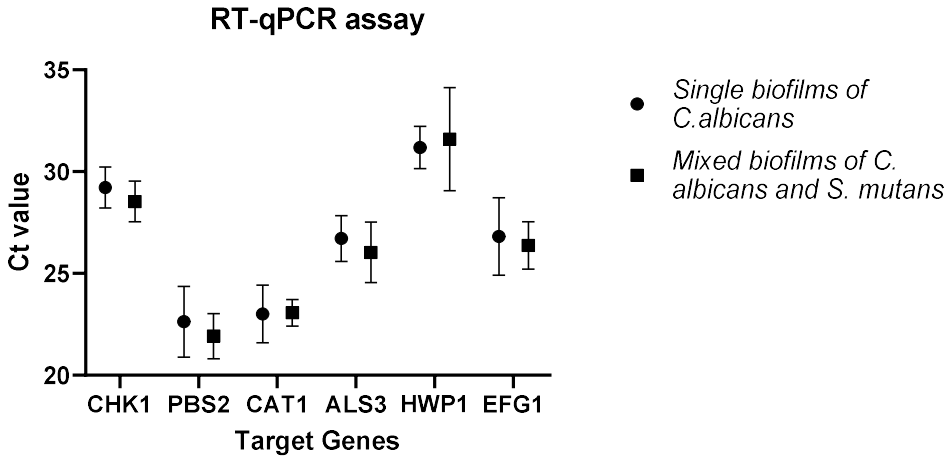



Figure 12. Comparison of virulence and quorum sensing gene expressions in single-species biofilms and 1:10 mixed-species biofilms as determined by RT-qPCR. Unpaired t-test did not show any significant difference for all the genes tested.

The interactions between *C. albicans* and *S. mutans* have been variously described as both synergistic and antagonistic (Sztajer et al. 2014; Kim et al. 2017). In a study conducted by Falsetta et. al. in 2014, it was reported that *C. albicans* triggers the development of virulence in *S. mutans*. Conversely, a different study by Willems et al. (2016) demonstrated that *C. albicans* diminishes the cariogenic potential of *S. mutans* (Willems et al., 2016). It is clear from different reports that the interactions between species can be affected by many variables including cell density, phase of cell growth, and nutrients in the growth medium. These factors could be the reasons why the results of this study are different compared with that of others and cautions the interpretation of the result.

# CHAPTER 5

# DISCUSSION

In this study, careful attention was given to ensuring an unbiased and equitable comparison of gene expressions in *S albicans* between single-species and *C. albicans/ S. mutans* mixed-species biofilms. To achieve this, effort was taken to design methodologies that minimize the variations of various procedures employed for comparative studies of the single species and mixed species biofilms. These involved the standardization, as far as possible, procedure such as the culture conditions, RNA extraction, cell quantification, etc.

Viable counts were employed to quantify the cell populations within the single-species and mixed-species biofilms. The results, as illustrated in Table 9 indicated that there were no significant differences in the counts of *C. albicans* cells between the single-species and 1:10 mixed-species biofilms ($p=0.69$). This finding provided assurance that an equal representation of *C. albicans* cells was present in both biofilm types, thereby enabling a reliable comparison of gene expressions between them. Based on these results, it was decided to select the 1:10 mixed-species biofilms for further investigation and comparison of gene expressions with single-species biofilms. This choice gave the assurance that of a fair assessment of gene expression variations between the single-species and mixed-species biofilms. By employing this approach, the study aimed to

minimize potential confounding factors and ensure as accurate an evaluation as feasible, of gene expression differences in the context of single-species and mixed-species biofilms.

In evaluating the results of the RT-qPCR analysis, it is important to consider various factors that could have influenced the observed lack of impact of *S. mutans* on the expression of *C. albicans* genes associated with key virulence factors. One plausible explanation for these findings could be the limited selection of genes for analysis. The chosen genes, HWP1, EFG1, ALS3, CAT1, CHK1, and PBS2, represent important proteins involved in various aspects of *C. albicans'* virulence expression, such as hyphal formation, cell wall construction, adherence, stress response, and regulation of virulence genes and biofilm formation. However, it is crucial to recognize that *C. albicans* possesses a complex network of genes and regulatory pathways that contribute to its virulence. Thus, the lack of significant impact observed in this study may be due to the exclusion of other genes that could have been influenced by *S. mutans*.

Additionally, the timing of the analysis and the specific experimental conditions employed could also influence the results. Virulence gene expression in *C. albicans* is known to be tightly regulated and can be influenced by various environmental cues, including the presence of other microorganisms. Therefore, the interaction between *S. mutans* and *C. albicans* may be dynamic and context-dependent, meaning that different results could be obtained under different conditions or at different time points. Furthermore, it is worth considering the

possibility of other mechanisms of interaction between *S. mutans* and *C. albicans* that were not investigated in this study. For example, *S. mutans* and *C. albicans* can form dual-species biofilms, which could lead to altered gene expression patterns compared to mono-species cultures. Exploring additional factors, such as metabolite exchange, intercellular signaling, or physical interactions between the two species, may provide further insights into their interaction and its impact on virulence gene expression.

While the limited selection of genes analyzed in the RT-qPCR study did not reveal a significant impact of *S. mutans* on the expression of *C. albicans* virulence genes, it is important to consider the potential influence of unexamined genes, the dynamic nature of their interaction, and the possibility of alternative mechanisms that were not explored. Further research using a broader gene panel, different experimental conditions, and exploring additional aspects of their interaction would be valuable to gain a comprehensive understanding of the interplay between *S. mutans* and *C. albicans* in terms of virulence expression.

Furthermore, the influence of *S. mutans* on *C. albicans* virulence expression may also be contingent upon population density. In the study, a fixed ratio of *C. albicans* to *S. mutans* of 1:10 was employed within the biofilm. However, this fixed ratio might have failed to account for potential interactions and their consequential effects on *C. albicans* virulence and quorum sensing. By overlooking such interactions between these two microorganisms, the true impact on *C. albicans'* gene expression might have been underestimated.

Another crucial factor to consider is the absence of interaction with the host. The interplay between the two microbes and the host, along with the environmental conditions in which they thrive, greatly influences their behavior and virulence. Given that the study was conducted in vitro, and the experimental conditions remained identical for both single-species and mixed-species biofilms, it becomes less likely for gene expression to demonstrate substantial changes.

To gain a more comprehensive understanding of the relationship between *S. mutans* and *C. albicans* in terms of virulence expression, further investigations are necessary. Conducting in vivo studies that more closely mimic the host environment would be crucial. Such studies would shed light on the complex interplay between the microorganisms, the host immune response, and the environmental factors influencing their behavior. Moreover, exploring the effects of different population densities, dynamic ratios, and the potential synergistic or antagonistic interactions between *S. mutans* and *C. albicans* would provide valuable insights into their combined virulence expression and quorum sensing mechanisms. By considering the multifaceted nature of these interactions, we can gain a more accurate understanding of the overall impact on gene expression and virulence of both microorganisms.

In recent study, Xiao et al. investigated the expression of virulence genes in the interactions between *C. albicans* and *S. mutans*, focusing on the formation of dual-species biofilms. They discovered that alterations in the development time of these biofilms led to the upregulation or downregulation of virulence

genes in both species (Xiao et al., 2023). Notably, they observed the upregulation of certain target virulence genes in *C. albicans*, including HWP1, CHT2, and SOD3, at various time points ranging from 24 to 52 hours. However, their study did not encompass the examination of quorum sensing genes, and their results demonstrated dynamic changes in gene expression over time. In contrast, our research focused on gene expression at a specific time point of 24 hours during biofilm development. To gain a more comprehensive understanding of the complex dynamics underlying these interactions and their impact on biofilm formation and virulence expression, it would be valuable for future studies to explore gene expression in mixed-species biofilms with different growth durations and ratios of *C. albicans* and *S. mutans*.

Hence, the interpretation of the RT-qPCR results should consider the limited gene selection, the potential influence of population density, time setting, and the absence of host interaction. A broader gene analysis, consideration of different microbial ratios, and exploring host-related conditions would provide a more comprehensive understanding of *S. mutans* impact on *C. albicans* virulence and quorum sensing.

# CHAPTER 6

# CONCLUSION

In an *in vitro* mixed-species biofilm model, *C. albicans* was not adversely affected by *S. mutans* in the expression of selected quorum sensing and virulence genes. Rather, it appeared that *S. mutans* could have significantly increased the growth of *C. albicans* in their co-habitation in a biofilm. Cell density may play an important role in the interaction between *C. albicans* and *S. mutans*.

Due to the controlled environment of in vitro experiments, which did not involve the presence of a host, this study excluded the influence of factors related to the immune system and the host. This omission may have contributed to the observed outcome, wherein *C. albicans*, known to exist as a commensal organism, did not undergo the transformation into a pathogenic state. Consequently, the study found that the expression of virulence factors by *C. albicans* did not undergo significant changes. By removing the complexities associated with the host's immune response and other host-related factors, the study isolated the impact of these specific elements on the virulence expression of *C. albicans*. Although the absence of the host context limits the generalizability of the findings to real-life scenarios, it allowed the researchers to focus on elucidating the intrinsic mechanisms within the fungus itself. It is important to note that the results obtained in this controlled setting may not fully

reflect the behavior of *C. albicans* in the presence of a host, where immune responses and other factors play a crucial role in the transition from commensalism to pathogenicity. Further research incorporating host factors is necessary to gain a comprehensive understanding of the virulence expression and pathogenic potential of *C. albicans* in a more realistic and complex biological context.

Recognising the important roles of *C. albicans* and *S. mutans* in oral mucosal infection and caries, respectively, and the significance of *C. albicans*' virulence, this study was undertaken to examine the effect of *S. mutans* on the growth and expression of virulence in *C. albicans*. It is anticipated that the results of this study can provide some additional input into wide knowledge base on the interspecies interaction between *S albicans* and *S mutans* in the mixed biofilm environment.

**Study limitations**

The results obtained from this study is not entirely reflective of in vivo *C. albicans* and *S. mutans* interactions as the biofilms examined were grown from ATCC strains of *S. mutans* and *C. albicans* cultured in the laboratory. The growth and survival traits of these reference strains are expected to differ to some degree from wild or clinical strains. Furthermore, the mixed-species biofilms were developed using only *C. albicans* and *S. mutans* ratios of 1:3 and 1:10. Other researchers have used ratios varying from 1:2 (Ellepola et al. 2017) to 1:

100 (Septiana et al. 2019). It is possible that different or more significant results could have been obtained with the use of a wider range of *C. albicans: S. mutans* ratios to make mixed-species biofilms. The measurement of total biomass in addition to viable counts would also have provided more information on the effect of *S. mutans* on the growth and viability of *C. albicans* in mixed biofilms.

**Future studies**

Further investigations are necessary to better understand the factors that affect interactions between *C. albicans* and *S. mutans* in mixed-species biofilms and the impact of these interactions on the pathogenesis of oral infections and dental caries. For instance, cells in mixed-species biofilms can be examined at different stages of biofilm formation to see whether gene expression varies with nutrient depletion or other changes in the aging biofilm population. Biofilm cells can also be studied for changes in their anti-bacterial or antifungal susceptibilities because of adaptation to co-habitation in the mixed-species biofilm.

## REFERENCES


Albuquerque, P. and Casadevall, A. (2012) 'Quorum sensing in fungi a review', Medical Mycology, pp. 337–345. Available at: https://doi.org/10.3109/13693786.2011.652201.

Alem, M.A.S. et al. (2006) 'Production of tyrosol by Candida albicans biofilms and its role in quorum sensing and biofilm development', Eukaryotic Cell, 5(10), pp. 1770–1779. Available at: https://doi.org/10.1128/EC.00219-06.

Arana, D.M. et al. (2005) 'The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen Candida albicans', Microbiology, 151(4), pp. 1033–1049. Available at: https://doi.org/10.1099/mic.0.27723-0.

Barriuso, J. et al. (2018) 'Role of quorum sensing and chemical communication in fungal biotechnology and pathogenesis', FEMS Microbiology Reviews. Oxford University Press, pp. 627–638. Available at: https://doi.org/10.1093/femsre/fuy022.

Bhattacharya, S., Sae-Tia, S. and Fries, B.C. (2020) 'Candidiasis and mechanisms of antifungal resistance', Antibiotics. MDPI AG, pp. 1–19. Available at: https://doi.org/10.3390/antibiotics9060312.

Brown, A.J.P., Odds, F.C. and Gow, N.A.R. (2007) 'Infection-related gene expression in Candida albicans', Current Opinion in Microbiology, pp. 307–313. Available at: https://doi.org/10.1016/j.mib.2007.04.001.

Brown, G.D. et al. (2012) 'Hidden killers: Human fungal infections', Science Translational Medicine. Available at: https://doi.org/10.1126/scitranslmed.3004404.

Calderone, R.A. and Fonzi, W.A., 2001. Virulence factors of Candida albicans. Trends in microbiology, 9(7), pp.327-335. Available at: https://doi.org/10.1016/S0966-842X(01)02094-7

Chakravarti, A. et al. (2017) 'The iron-dependent regulation of the candida albicans oxidative stress response by the CCAAT-binding factor', PLoS ONE, 12(1). Available at: https://doi.org/10.1371/journal.pone.0170649.

Cheetham, J. et al. (2007) 'A Single MAPKKK Regulates the Hog1 MAPK Pathway in the Pathogenic Fungus Candida albicans', Molecular Biology of the Cell, 18, pp. 4603–4614. Available at: https://doi.org/10.1091/mbc.E07-06.

D'Enfert, C. et al. (2021) 'The impact of the fungus-host-microbiota interplay upon Candida albicans infections: Current knowledge and new perspectives',

FEMS Microbiology Reviews. Oxford University Press. Available at: https://doi.org/10.1093/femsre/fuaa060.

Dimopoulos, G. et al. (2008) ‘Candida albicans versus non-albicans intensive care unit-acquired bloodstream infections: Differences in risk factors and outcome’, Anesthesia and Analgesia, 106(2), pp. 523–529. Available at: https://doi.org/10.1213/ane.0b013e3181607262.

dos Santos, J.D. et al. (2020) ‘Streptococcus mutans Secreted Products Inhibit Candida albicans Induced Oral Candidiasis’, Frontiers in Microbiology, 11(July), pp. 1–17. Available at: https://doi.org/10.3389/fmicb.2020.01605.

Ellepola, K. et al. (2017) ‘Bacterial GtfB Augments Candida albicans Accumulation in Cross-Kingdom Biofilms’, Journal of Dental Research, 96(10), pp. 1129–1135. Available at: https://doi.org/10.1177/0022034517714414.

Falsetta, M.L. et al. (2014) ‘Symbiotic relationship between Streptococcus mutans and Candida albicans synergizes virulence of plaque biofilms in vivo’, Infection and Immunity, 82(5), pp. 1968–1981. Available at: https://doi.org/10.1128/IAI.00087-14.

Farkas, D.H. and Holland, C.A. (2009) Overview of Molecular Diagnostic Techniques and Instrumentation, Cell and Tissue Based Molecular Pathology: A Volume in the Series Foundations in Diagnostic Pathology. Available at: https://doi.org/10.1016/B978-0-443-06901-7.50008-0.

Hedges, A.J., Shannon, R. and Hobbs, R.P. (1978) Comparison of the Precision Obtained in Counting Viable Bacteria by the Spiral Plate Maker, the Droplette and the Miles & Misra Methods, Journal of Applied Bacteriology.

Hornby, J.M. et al. (2001) ‘Quorum Sensing in the Dimorphic Fungus Candida albicans Is Mediated by Farnesol’, Applied and Environmental Microbiology, 67(7), pp. 2982–2992. Available at: https://doi.org/10.1128/AEM.67.7.2982-2992.2001.

Jugder, B.E. et al. (2022) ‘Vibrio cholerae high cell density quorum sensing activates the host intestinal innate immune response’, Cell Reports, 40(12). Available at: https://doi.org/10.1016/j.celrep.2022.111368.

Kim, D. et al. (2017) ‘Candida albicans stimulates Streptococcus mutans microcolony development via cross-kingdom biofilm-derived metabolites’, Scientific Reports, 7. Available at: https://doi.org/10.1038/srep41332.

Kruppa, M. (2009) ‘Quorum sensing and Candida albicans’, Mycoses, pp. 1–10. Available at: https://doi.org/10.1111/j.1439-0507.2008.01626.x.

Kumamoto, C.A. and Vinces, M.D. (2005) 'Contributions of hyphae and hypha-co-regulated genes to Candida albicans virulence', Cellular Microbiology, 7(11), pp. 1546–1554. Available at: https://doi.org/10.1111/j.1462-5822.2005.00616.x.

Lassak, T. et al. (2011) 'Target specificity of the Candida albicans Efg1 regulator', Molecular Microbiology, 82(3), pp. 602–618. Available at: https://doi.org/10.1111/j.1365-2958.2011.07837.x.

Leitão, J.H. (2020) 'Microbial virulence factors', International Journal of Molecular Sciences. MDPI AG, pp. 1–6. Available at: https://doi.org/10.3390/ijms21155320.

Li, S. et al. (2022) 'Biofilm matrix cloaks bacterial quorum sensing chemo attractants from predator detection', The ISME Journal, 16(5), pp. 1388–1396. Available at: https://doi.org/10.1038/s41396-022-01190-2.

Liu, Y. and Filler, S.G. (2011) 'Candida albicans Als3, a multifunctional adhesin and invasin', Eukaryotic Cell, 10(2), pp. 168–173. Available at: https://doi.org/10.1128/EC.00279-10.

Maras, B. et al. (2021) 'Hyperexpression of CDRs and HWP1 genes negatively impacts on Candida albicans virulence', PLoS ONE, 16(6 June), pp. 1–12. Available at: https://doi.org/10.1371/journal.pone.0252555.

McIlwain, D.R., Berger, T. and Mak, T.W. (2013) 'Caspase functions in cell death and disease', Cold Spring Harbor Perspectives in Biology, 5(4), pp. 1–28. Available at: https://doi.org/10.1101/cshperspect.a008656.

Naglik, J.R., Challacombe, S.J. and Hube, B. (2003) 'Candida albicans Secreted Aspartyl Proteinases in Virulence and Pathogenesis', Microbiology and Molecular Biology Reviews, 67(3), pp. 400–428. Available at: https://doi.org/10.1128/mmbr.67.3.400-428.2003.

Nailis, H. et al. (2006) 'Development and evaluation of different normalization strategies for gene expression studies in Candida albicans biofilms by real-time PCR', BMC Molecular Biology, 7. Available at: https://doi.org/10.1186/1471-2199-7-25.

Nealson, K.H., Platt, T. and Hastings, J.W. (1970) Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System1, JOURNAL OF BACTERIOLOGY. Available at: https://journals.asm.org/journal/jb.

Noble, S.M., Gianetti, B.A. and Witchley, J.N. (2017) 'Candida albicans cell-type switching and functional plasticity in the mammalian host', Nature Reviews Microbiology, 15(2), pp. 96–108. Available at: https://doi.org/10.1038/nrmicro.2016.157.

Peters, B.M. et al. (2012) ‘Polymicrobial interactions: Impact on pathogenesis and human disease’, Clinical Microbiology Reviews, pp. 193–213. Available at: https://doi.org/10.1128/CMR.00013-11.

Sharkey, L.L. et al. (1999) ‘HWP1 functions in the morphological development of Candida albicans downstream of EFG1, TUP1, and RBF1’, Journal of Bacteriology, 181(17), pp. 5273–5279. Available at: https://doi.org/10.1128/jb.181.17.5273-5279.1999.

Schröppel, K. et al. (2000) Repression of Hyphal Proteinase Expression by the Mitogen-Activated Protein (MAP) Kinase Phosphatase Cpp1p of Candida albicans Is Independent of the MAP Kinase Cek1p, INFECTION AND IMMUNITY.

Sebaa, S., Boucherit-Otmani, Z. and Courtois, P. (2019) ‘Effects of tyrosol and farnesol on Candida albicans biofilm’, Molecular Medicine Reports, 19(4), pp. 3201–3209. Available at: https://doi.org/10.3892/mmr.2019.9981.

Septiana, S. et al. (2019) ‘Cajuputs candy impairs Candida albicans and Streptococcus mutans mixed biofilm formation in vitro’, F1000Research, 8, p. 1923. Available at: https://doi.org/10.12688/f1000research.20700.1.

Shirtliff, M.E. et al. (2009) ‘Farnesol-induced apoptosis in Candida albicans’, Antimicrobial Agents and Chemotherapy, 53(6), pp. 2392–2401. Available at: https://doi.org/10.1128/AAC.01551-08.

Sztajer, H. et al. (2014) ‘Cross-feeding and interkingdom communication in dual-species biofilms of Streptococcus mutans and Candida albicans’, ISME Journal, 8(11), pp. 2256–2271. Available at: https://doi.org/10.1038/ismej.2014.73.

Tong, Y. and Tang, J. (2017) ‘Candida albicans infection and intestinal immunity’, Microbiological Research. Elsevier GmbH, pp. 27–35. Available at: https://doi.org/10.1016/j.micres.2017.02.002.

Willems, H.M. et al. (2016) ‘Candida albicans in oral biofilms could prevent caries’, Pathogens and disease, 74(5). Available at: https://doi.org/10.1093/femspd/ftw039.

Winzer, K. and Williams, P. (2001) ‘Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria’, International Journal of Medical Microbiology, 291(2), pp. 131–143. Available at: https://doi.org/10.1078/1438-4221-00110.

Wysong, D.R. et al. (1998) Cloning and Sequencing of a Candida albicans Catalase Gene and Effects of Disruption of This Gene †, INFECTION AND IMMUNITY.

Xu, H. et al. (2014) ‘Streptococcal co-infection augments candida pathogenicity by amplifying the mucosal inflammatory response’, Cellular Microbiology, 16(2), pp. 214–231. Available at: https://doi.org/10.1111/cmi.12216.

Xiao, J. et al. (2023) ‘Dual transcriptome of Streptococcus mutans and Candida albicans interplay in biofilms’, Journal of Oral Microbiology, 15(1). Available at: https://doi.org/10.1080/20002297.2022.2144047.

Yano, J. et al. (2019) ‘Current patient perspectives of vulvovaginal candidiasis: Incidence, symptoms, management and post-treatment outcomes’, BMC Women’s Health, 19(1). Available at: https://doi.org/10.1186/s12905-019-0748-8.

Zhou, Y. et al. (2021) ‘Candida albicans CHK1 gene from two-component system is essential for its pathogenicity in oral candidiasis’, APPLIED MICROBIAL AND CELL PHYSIOLOGY [Preprint]. Available at: https://doi.org/10.1007/s00253-021-11187-0/Published.

# APPENDICES

## Appendix A

**Composition of TSB (BD Tryptic Soy Broth):**

Per Liter Purified Water

Tryptone (Pancreatic Digest of Casein) 17.0 g

Soytone (Peptic Digest of Soybean) 3.0 g

Glucose (= Dextrose) 2.5 g

Sodium Chloride 5.0 g

Dipotassium Phosphate 2.5 g

pH 7.3 ± 0.2 at 25°C

## Appendix B

**Composition of SDB (Sabouraud Dextrose Broth) (Himedia, India)**

Per Liter Purified Water

Pancreatic Digest of Casein 5.0 g

Peptic Digest of Animal Tissue 5.0 g

Dextrose 20.0 g

Final pH 5.6 ± 0.2 at 25°C

## Appendix C

**Composition of BHI broth (Himedia, India)**

Per Liter Purified Water

Calf brain, infusion from 200.0 g

Beef heart, infusion from 250.0 g

Proteose peptone 10.0 g

Dextrose 2.0 g

Sodium chloride 5.0 g

Disodium phosphate 2.5 g

Final pH 7.4 ± 0.2 at 25°C

## Appendix D

**BHI agar (Himedia, India)**

Per Liter Purified Water

HM infusion powder 12.5 g

BHI powder 5.0 g

Proteose peptone 10.0 g

Dextrose (Glucose) 2.0 g

Sodium chloride 5.0 g

Disodium phosphate 2.5.0 g

Agar 15.0 g

Final pH 7.4 ± 0.2 at 25°C

## Appendix E

**CHROMagar (CHROMagar Candida, USA, Difco)**

Per Liter Purified Water

Peptone 8.0 g

Sodium chloride 5.0 g

Sodium deoxycholate 1.0 g

Chromogenic mix 1.5 g

Polypropylene glycol 10.5 g

Agar 15.0 g

pH 7.4 ± 0.2 at 25°C

## Appendix F

### Preparation of TSB

1. Add 30.0 g of components to distilled water and bring the volume to 1.0 L. Mix thoroughly.
2. Shake to dissolve broth before dispensing into bottles or flasks. Autoclave for 15 minutes at 121°C.
3. Cool to 45°–50°C.

## Appendix G

### Preparation of CHROMagar

1. Add 47.7g of components to distilled water and bring the volume to 1.0 L. Mix thoroughly.
2. Heat in a boiling water bath or in a current of steam while shaking from time to time.
3. The medium is totally suspended if no visual particles stick to the glass wall.
4. The medium should not be heat treated further.
5. Complete dissolution with shaking in 5-minute sequences for approximately 35–40 minutes.
6. Do not autoclave or overheat.
7. Cool as fast as possible to 45°–50°C while gently shaking from time to time.
8. Pour into sterile Petri dishes.
9. To prevent any precipitate or clotting of the chromogenic mix in the plates, place Petri dishes during pouring procedure on a cool (max. 25°C) surface.

## Appendix H

### Preparation of BHI agar

1. Add 47.0 g of components to distilled water and bring the volume to 1.0 L. Mix thoroughly.
2. Shake to dissolve agar before dispensing into bottles or flasks. Autoclave for 15 minutes at 121°C.
3. Cool to 45°–50°C.
4. Pour into sterile Petri dishes.
5. To prevent any precipitate or clotting of the chromogenic mix in the plates, place Petri dishes during pouring procedure on a cool (max. 25°C) surface.

## Appendix I

### Preparation of BHI broth

1. Add 37.0 g of components to distilled water and bring the volume to 1.0 L. Mix thoroughly.
2. Shake to dissolve broth before dispensing into bottles or flasks. Autoclave for 15 minutes at 121°C.
3. Cool to 45°–50°C.

## Appendix J

### Preparation of SDA

1. Add 65.0 g of components to distilled water and bring the volume to 1.0 L. Mix thoroughly.
2. Shake to dissolve agar before dispensing into bottles or flasks. Autoclave for 15 minutes at 121°C.
3. Cool to 45°–50°C.
4. Pour into sterile Petri dishes.

## Appendix K

**Preparation of SDB**

1. Add 30.0 g of components to distilled water and bring the volume to 1.0 L. Mix thoroughly.

2. Shake to dissolve broth before dispensing into bottles or flasks. Autoclave for 15 minutes at 121°C.

3. Cool to 45°–50°C.

## Appendix L

**Summary results for the expression of quorum sensing and virulence genes in single and mixed-species biofilms**

**The numbers in the forms stand for quantification values in RT-qPCR. Efficiency means whether the amplification of target sequence is duplicated at 100%. Normally, it should range from 90% to 100%, or from 0.9 to 1.**

**Table A. Expression of Housekeeping genes in biofilms (CT value)**

| Gene | ACT1 | PMA1 | RPP2B |
|---|---|---|---|
| Single-1 | 18.45 | 22.67 | 19.71 |
| Single-2 | 20.26 | 25.15 | 21.58 |
| Single-3 | 20.83 | 23.96 | 23.4 |
| Mixed-1 | 17.84 | 20.25 | 20.77 |
| Mixed-2 | 20.22 | 22.92 | 22.45 |
| Mixed-3 | 20.92 | 25.13 | 21.32 |
| Efficiency | 1.07 | 0.91 | 0.93 |

## Appendix M

**Table B. Expression of Quorum sensing genes in biofilms (CT value)**

| Gene | CHK1 | PBS2 |
|---|---|---|
| Single-1 | 27.97 | 20.47 |
| Single-2 | 29.64 | 23.22 |
| Single-3 | 30.04 | 24.29 |
| Mixed-1 | 27.36 | 20.49 |
| Mixed-2 | 28.68 | 22.73 |
| Mixed-3 | 29.56 | 22.52 |
| Efficiency | 0.71 | 0.9 |

## Appendix N

**Table C. Expression of Virulence genes in biofilms (CT value)**

| Gene | HWP1 | EFG1 | CAT1 | ALS3 |
|---|---|---|---|---|
| Single-1 | 29.87 | 23.5 | 21.51 | 25.29 |
| Single-2 | 31.96 | 26.86 | 22.87 | 27.41 |
| Single-3 | 31.71 | 27.39 | 24.64 | 27.44 |
| Mixed-1 | 28.61 | 24.615 | 22.56 | 24.11 |
| Mixed-2 | 32 | 27.015 | 23.89 | 26.96 |
| Mixed-3 | 34.17 | 25.135 | 22.74 | 27.01 |
| Efficiency | 0.96 | 1.1 | 0.9 | 0.94 |

## Appendix O

**Table D. Comparison of expression of CHK1 between single and mixed biofilms (CT value)**

| Gene | CHK1 | ACT 1 | Intermediate value target | Intermediate value ref | target/ref | P |
|---|---|---|---|---|---|---|
| Single-1 | 27.97 | 18.45 | 3.04158E-07 | 1.48029E-06 | 0.2055 | 0.437 |
| Single-2 | 29.64 | 20.26 | 1.24164E-07 | 3.9668E-07 | 0.313 | |
| Single-3 | 30.04 | 20.83 | 1.00184E-07 | 2.62022E-07 | 0.3823 | |
| Mixed-1 | 27.36 | 17.84 | 4.21916E-07 | 2.30722E-06 | 0.1829 | |
| Mixed-2 | 28.68 | 20.22 | 2.07812E-07 | 4.08394E-07 | 0.5089 | |
| Mixed-3 | 29.56 | 20.92 | 1.29609E-07 | 2.45415E-07 | 0.5281 | |
| Efficiency | 0.71 | 1.07 | | | | |

## Appendix P

**Table E. Comparison of expression of PBS2 gene between single and mixed biofilms (CT value)**

| Gene | PBS2 | ACT1 | Intermediate value target | Intermediate value ref | target/ref | p |
|---|---|---|---|---|---|---|
| Single-1 | 20.47 | 18.45 | 1.9675E-06 | 1.48029E-06 | 1.329128 | 0.396979 |
| Single-2 | 23.22 | 20.26 | 3.36776E-07 | 3.9668E-07 | 0.848986 | |
| Single-3 | 24.29 | 20.83 | 1.80697E-07 | 2.62022E-07 | 0.689625 | |
| Mixed-1 | 20.49 | 17.84 | 1.9424E-06 | 2.30722E-06 | 0.841879 | |
| Mixed-2 | 22.73 | 20.22 | 4.61244E-07 | 4.08394E-07 | 1.129408 | |
| Mixed-3 | 22.52 | 20.92 | 5.27799E-07 | 2.45415E-07 | 2.150644 | |

## Appendix Q

**Table F. Comparison of expression of EFG1 gene between single and mixed biofilms (CT value)**

| Gene | EFG1 | ACT1 | Intermediate value target | Intermediate value ref | target/ref | p |
|---|---|---|---|---|---|---|
| Single-1 | 23.5 | 18.45 | 2.67822E-08 | 1.48029E-06 | 0.018093 | 0.689401 |
| Single-2 | 26.86 | 20.26 | 2.21406E-09 | 3.9668E-07 | 0.005581 | |
| Single-3 | 27.39 | 20.83 | 1.49422E-09 | 2.62022E-07 | 0.005703 | |
| Mixed-1 | 24.615 | 17.84 | 1.17104E-08 | 2.30722E-06 | 0.005076 | |
| Mixed-2 | 27.015 | 20.22 | 1.97354E-09 | 4.08394E-07 | 0.004832 | |
| Mixed-3 | 25.135 | 20.92 | 7.96192E-09 | 2.45415E-07 | 0.032443 | |
| Efficiency | 1.1 | 1.07 | | | | |

## Appendix R

**Table G. Comparison of expression of HWP1 gene between single and mixed biofilms (CT value)**

| Gene | HWP1 | ACT 1 | Intermediate value target | Intermediate value ref | target/ref | P |
|---|---|---|---|---|---|---|
| Single-1 | 29.87 | 18.45 | 1.86342E-09 | 1.48029E-06 | 0.001259 | 0.521631 |
| Single-2 | 31.96 | 20.26 | 4.56558E-10 | 3.9668E-07 | 0.001151 | |
| Single-3 | 31.71 | 20.83 | 5.40207E-10 | 2.62022E-07 | 0.002062 | |
| Mixed-1 | 28.61 | 17.84 | 4.35065E-09 | 2.30722E-06 | 0.001886 | |
| Mixed-2 | 32 | 20.22 | 4.44432E-10 | 4.08394E-07 | 0.001088 | |
| Mixed-3 | 34.17 | 20.92 | 1.03183E-10 | 2.45415E-07 | 0.00042 | |
| Efficiency | 0.96 | 1.07 | | | | |

## Appendix S

**Table H. Comparison of expression of ALS3 gene between single and mixed biofilms (CT value)**

| Gene | ALS3 | ACT1 | Intermediate value target | Intermediate value ref | target/ref | p |
|---|---|---|---|---|---|---|
| Single-1 | 25.29 | 18.45 | 5.26616E-08 | 1.48029E-06 | 0.035575 | 0.17837 |
| Single-2 | 27.41 | 20.26 | 1.29227E-08 | 3.9668E-07 | 0.032577 | |
| Single-3 | 27.44 | 20.83 | 1.26684E-08 | 2.62022E-07 | 0.048348 | |
| Mixed-1 | 24.11 | 17.84 | 1.15107E-07 | 2.30722E-06 | 0.04989 | |
| Mixed-2 | 26.96 | 20.22 | 1.74127E-08 | 4.08394E-07 | 0.042637 | |
| Mixed-3 | 27.01 | 20.92 | 1.68452E-08 | 2.45415E-07 | 0.06864 | |
| Efficiency | 0.94 | 1.07 | | | | |

## Appendix T

**Table I. Comparison of expression of CAT1 gene between single and mixed biofilms (CT value)**

| Gene | CAT1 | ACT1 | Intermediate value target | Intermediate value ref | target/ref | p |
|---|---|---|---|---|---|---|
| Single-1 | 21.51 | 18.45 | 6.4474E-07 | 1.48029E-06 | 0.4355496 | 0.833 |
| Single-2 | 22.87 | 20.26 | 2.61803E-07 | 3.9668E-07 | 0.6599843 | |
| Single-3 | 24.64 | 20.83 | 8.1015E-08 | 2.62022E-07 | 0.3091916 | |
| Mixed-1 | 22.56 | 17.84 | 3.21509E-07 | 2.30722E-06 | 0.139349 | |
| Mixed-1 | 23.89 | 20.22 | 1.33173E-07 | 4.08394E-07 | 0.3260897 | |
| Mixed-1 | 22.74 | 20.92 | 2.85357E-07 | 2.45415E-07 | 1.1627552 | |
| Efficiency | 0.9 | 1.07 | | | | |

## Appendix U

### RNA extraction

Preparation and reagents:

a: Mix 950µl buffer RLT and 9.5µl β-mercaptoethanol in a 1.5ml tube.

b: Prepare 1200µl 70% ethanol (840µl ethanol + 360µl H2O) in a 1.5ml tube.

c: Bring buffer RDD and DNase1 to room temperature.

1. Add 900µl DNA/RNA shield into wells to suspend biofilm cells with the aid

of a cell scraper. Pipette the suspension into a 1.5 ml tube.

2. Centrifuge at 13000 x g for 1 minute. Discard the supernatant.
3. Add the prepared buffer RLT and β-mercaptoethanol to the pellet and vortex to resuspend. Pipette the suspension into a bashing bead tube and vortex for 5 minutes.
4. Centrifuge at 13000 x g for 1 minute.
5. Pipette 600µl of the supernatant into a QIA shredder (purple column).
6. Centrifuge at 13000 x g for 1 minute.
7. Remove the column and add 600µl of 70% ethanol to the flowthrough. Mix well then transfer 600µl into a pink column in a new centrifuge tube.
8. Centrifuge at 13000 x g for 1 minute. Discard the flowthrough.
9. Pipette the remaining 600µl from step 7 into the pink column.
10. Centrifuge at 13000 x g for 1 minute. Discard the flowthrough.
11. Pipette 350µl buffer RW1 into the pink column.
12. Centrifuge at 13000 x g for 1 minute. Discard the flowthrough.
13. Mix 70µl RDD buffer with 10µl DNase1 and pipette directly onto the membrane of the column. Incubate at room temperature for 15 minutes. Discard the flowthrough.
14. Pipette 350µl buffer RW1 into the pink column.
15. Centrifuge at 13000 x g for 1 minute. Discard the flowthrough.
16. Add 500µl buffer RPE to the column.
17. Centrifuge at 13000 x g for 1 minute. Discard the flowthrough.
18. Add 500µl buffer RPE to the column.
19. Centrifuge at 13000 x g for 2 minutes. Discard the flowthrough. Transfer the column to a new centrifuge tube.

20. Centrifuge at 16000 x g for 1 minute to dry.
21. Transfer the column into a new 1.5ml tube.
22. Pipette 30µl RNase -free water directly onto the membrane and incubate for 1 minute at room temperature.
23. Centrifuge at 13000 x g for 1 minute.
24. RNA in the tube below, keep it in -80℃, take 3µl to test nanodrop RNA.

## Appendix V

**RNA Clean Up**

Procedures

A. DNase Digestion of RNA before RNA Clean Up

   a. Reagents

      1. 10 µl Buffer RDD
      2. 2.5 µl of DNase1
      3. Up to 87.5 µl RNA

   b. Steps

      1. Mix all 3 reagents on the above in a microcentrifuge tube and incubate at room temperature for 15 minutes.

B. RNA Clean Up

   Reagents

      1. Mix 200 µl Buffer RPE with 800 µl of absolute ethanol
      2. DNase1 incubation mix (for on-column treatment)
         I. Pipette 70 µl of buffer RDD into 10 µl of DNase

Steps

1. Pipette 350 µl of buffer RLT into reagents prepared in DNase digestion and mix by pipetting.
2. Pipette 250 µl of ethanol to the tube and mix by pipetting.
3. Transfer 700 µl to an RNeasy spin column placed in a 2 ml collection tube.
4. Centrifuge at 13000 x g for 1 minute.
5. Discard the flowthrough and collection tube.
6. Transfer the RNeasy spin column to a new collection tube and perform on-column DNase digestion
    I. Pipette 350 µl of RW1 into RNeasy Mini Spin Column and centrifuge at 13000 x g for 1 minute.
    II. Pipette 80 µl of the DNase1 incubation mix onto RNeasy spin column membrane.
    III. Incubate for 15 minutes at room temperature.
    IV. Pipette 350 µl of RW1 to RNeasy spin column and centrifuge for 15s at 10000 x g. Discard the flowthrough.
7. Add 500 µl of Buffer RPE into the RNeasy spin column and centrifuge at 13000 x g for 1 minute. Discard the flowthrough.
8. Add 500 µl of Buffer RPE to the RNeasy spin column and centrifuge at 13000 x g for 2 minutes. Discard the flowthrough and tube.

9. Place the RNeasy spin column in a new collection tube and centrifuge at 13000 x g for 1 minute to dry the membrane.
10. Place the RNeasy spin column in a new 1.5 ml collection tube and add 30 µl of RNase-free water into the RNeasy spin column.
11. Incubate at room temperature for 1 minute.
12. Centrifuge at 13000 x g for 1 minute.
13. For the second elution, place the RNeasy spin column in a new 1.5 ml tube and add in 30 µl of RNase-free water into the RNeasy spin column.
14. Incubate at room temperature for 1 minute.
15. Centrifuge at 13000 x g for 1 minute.

## Appendix W

**Reverse transcription**

Preparation

1. The entire procedure is conducted on ice.
2. Set the thermal cycler parameters.
3. Dilute RNA according to the table below.

| Items | RNA µl | $H_2O$ µl | Nowµl | 4×DNmm(Gdnar) µl | 5×RTmmIIµl | Totalµl |
|---|---|---|---|---|---|---|
| C. albicans-1 | 2.2 | 17.8 | 20 | 6.7 | 6.7 | 33.4 |
| C. albicans-2 | 8.1 | 11.9 | 20 | 6.7 | 6.7 | 33.4 |
| C. albicans-3 | 2.8 | 17.2 | 20 | 6.7 | 6.7 | 33.4 |
| Mixed-1 | 2.6 | 17.4 | 20 | 6.7 | 6.7 | 33.4 |
| Mixed-2 | 2.4 | 17.6 | 20 | 6.7 | 6.7 | 33.4 |
| Mixed-3 | 20 | 0 | 20 | 6.7 | 6.7 | 33.4 |
| RT-con-C. a | 1 | 5 | 6 | 2 | (noRTmm) 2 | 10 |
| Std-cv-C. a | 4 | 8 | 12 | 4 | 4 | 20 |

4. Denature RNA at 65ºC for 5 minutes then immediately place on ice.
5. Add 4X DN master mix with gDNA remover and incubate at 37ºC for 5 minutes the immediately place on ice.
6. Add RT master mix II then incubate at 37ºC for 15 minutes, 50ºC for 5 minutes followed by 98ºC for 5 minutes.
7. Store cDNA at -20ºC.

## Appendix X

**Miles and Misra method for CFU (colony forming units)**

1. Materials:

   A calibrated dropping pipette, or automatic pipette, delivering drops of 10µl.

   Petri dishes containing nutrient agar.

   Phosphate Buffered Saline (PBS).

   Microorganism suspension.

2. The inoculum / suspension is serially diluted by adding 1x of suspension to 9x of diluent.

   Three agar plates are needed for each dilution series, for statistical reasons an average of at least 3 counts are needed.

3. The surface of the plates need to be sufficiently dry to allow a 10µl drop to be absorbed in 15–20 minutes.

4. Plates are divided into equal sectors (it is possible to use up to 6 per plate). The sectors are labelled with the dilutions.

5. In each sector, 1 x 10 µl drop of the appropriate dilution is dropped onto the surface of the agar and the drop is allowed to spread naturally. It is important to avoid touching the surface of the agar with the pipette.

6. The plates are left upright in the biosafety cabinet to dry before inversion and incubation at 37°C for 24 hours.

7. Each sector is observed for growth. Colonies are counted in the sector where the highest number of full-size discrete colonies can be seen (usually sectors containing between 2-20 colonies are counted).

8. The following equation is used to calculate the number of colony forming units (CFU) per ml from the original aliquot / sample:

CFU per ml = Average number of colonies for a dilution x 100 x dilution factor.

## Appendix Y

**Design PCR primers and check them for specificity.**

A. Designing primers for a known DNA sequence.

1. Go to the Primer BLAST submission form.
2. Enter the target sequence in FASTA format in the PCR Template section of the form.
3. In the primer pair specificity checking parameters section, select the appropriate source organism and the smallest database that is likely to contain the target sequence. These settings give the most precise results. For broadest coverage, choose the nr database and do not specify an organism.
5. Click the "Get Primers" button to submit the search and retrieve specific primer pairs.

B. Checking specificity of primers.

1. Go to the NCBI Primer BLAST submission form.
2. Enter both primer sequences in the Primer Parameters section of the form.
3. In the Primer Pair Specificity Checking Parameters section, select the appropriate source organism and the smallest database that is likely to contain the target sequence. These settings give the most precise results. For broadest coverage, choose the nr database and do not specify an organism.
4. Click the "Get Primers" button to submit the search and retrieve template and specificity information.