Intermicrobial Interactions in Prosthetic Speech Valve Biofilms

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DECLARATION

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any other qualification at this or any other university.

ABSTRACT

Treatment of advanced head and neck cancers sometimes requires laryngectomy which extends the patient's life span but makes natural speech impossible. Insertion of a silicone tracheoesophageal speech valve (TESV) can restore functional speech. This prosthesis frequently develops biofilm, causing valve obstruction and failure which will lead to infection to the wearer. Biofilms isolated from TESVs are usually polymicrobial with *Candida albicans* proposed as the main problematic agent. This study aimed to isolate, identify and model the biofilm community of TESVs. It is anticipated that improved understanding of intermicrobial interactions in TESV biofilms will lead to new approaches for biofilm control.

Ten TESV biofilms were collected and microorganisms identified using 'microbial culturomics' involving standard culture techniques, biochemical tests, microscopy, mass spectrometry and next generation sequencing. *C. albicans* was always present; and often with at least one other fungal species together with a complex bacterial population that included H_2O_2-producing *Lactobacillus* spp. Possibly related to this, we found some TESV derived *C. albicans* spp. to be relatively resistant to peroxide-based reactive oxygen species.

Species from a single TESV were tested for co-aggregation and an *in vitro* static biofilm model was developed to reflect the typical TESV biofilm composition. This model was used to assess the ability of different fungi and bacteria to compete within biofilms using quantitative PCR. Bacteria in the biofilm have a significant effect on the composition of *Candida* spp. and also impacted on the activation of the Hog1 stress-activated protein kinase in *Candida* spp.

In conclusion, this is the first study of TESV biofilms employing microbial culturomics for identification and provides evidence that the composition is more complex than a simple candidal infection. This study is also the first to show that *C. albicans* Hog1 activation is inhibited in polymicrobial biofilms containing clinically relevant bacteria.

In memory of

My father; Abdullah Hashim, My Grandma; Wan Kiah Wan Nan and My aunt; Wan Mashitoh Wan Ismail

"My Lord! Bestow on them Your Mercy as they did bring me up when I was young."

Al-Isra verse 24

ACKNOWLEDGEMENT

ABBREVIATIONS

°C	Degrees Celsius
•OH	hydroxyl radical
$^1O_2$	singlet oxygen
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AGE	Agarose gel electrophoresis
AS	Artificial saliva
BHYE	Brain heart yeast infusion broth
BSA	Bovine albumin serum
CFU	Colony forming unit
CLSM	Confocal Laser Scanning Microscopy
CO_2	Carbon dioxide
ConA	Concanavalin A
CSP	Competence stimulating peptide
CV	Crystal violet
CW	Calcofluor White
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extracellular matrix
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ENT	Ear, nose, and throat
EPS	Extracellular polymeric substances
et. al	*et alii* / and others
FAA	Fastidious anaerobe agar
FAM	Fluorescein amidite
FCS	Fetal calf serum
FE-SEM	Field emission scanning electron microscopy
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
gDNA	Genomic DNA
h	Hour
H_2O_2	Hydrogen peroxide
HBF	High biofilm former
HCl	Hydrochloric acid
Hwp1	Hyphal wall protein-1
Kb	Kilobase
KDa	Kilodalton
L	Litre

LB	Luria Bertani
LBF	Low biofilm former
LPS	Lipopolysaccharide
M	Molar
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight Mass Spectrophotometry
MGS	Mitis group streptococci
min	Minute
mm	Millimetre
mM	Millimolar
MRD	Modified Robbins Device
MW	Molecular weight
NAC	Non-*Albicans Candida*
NCBI	National Centre for Biotechnology Information
NGS	Next generation sequencing
NHS	National health service
NucB	Nuclease B
O_2^-	superoxide
O_2,	oxygen
OD	Optical density
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PK	Proteinase K
PLB	Protein lysis buffer
PMSF	Phenylmethyl sulphonyl floride
PNA	Peptide nucleic acid
qPCR	Qualitative polymerase chain reaction
QS	Quorum sensing
ROS	Reactive oxygen species
rpm	Revolutions per minute
RTF	Reduced transport fluid
s	Second
SD	Standard deviation
SDA	Saboraud dextrose agar
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
spp	Species
TEMED	Tetramethylethylenediamine
TESV	Tracheoesophageal speech valve

TSB	Tryptic soy broth
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
μ	Micro
μL	Microlitre
μm	Micrometre
μM	Micromolar

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Chapter 1. Literature review

1.1 Introduction

A variety of medical devices have been invented to help in patient management and improve quality of life. Silicone rubber is widely used as a biomaterial to construct various medical devices and became commercially available in the1940s after the work of Hyde (Dow Corning) and Rochow (General Electric). Hyde demonstrated the thermal stability and high electrical resistance of silicone resins and Rochow was was the first to derive silicones from silicon and methylchloride (Colas and Curtis, 2004). Current medical devices composed of silicone rubber include urinary catheters, central venous catheters, peritoneal dialysis catheters, intrauterine devices, endotracheal tubes, prosthetic joints, voice prostheses, and tissue fillers including, but not restricted to, breast implants, mechanical heart valves and pacemakers. The use of silicone as a biomaterial has many advantages that are generally related to its chemical properties; it is relatively hydrophobic, possesses low surface tension, is chemically inert and has good thermal stability.

One major disadvantage of introducing silicone (or any other biomaterial) within the body is that it is prone to colonisation by microorganisms and the subsequent formation of biofilms. The problem of biofilm development and device infection (biofouling) on urinary catheters, joint prostheses, prosthetic cardiac valves, central venous catheters and voice prostheses has been reviewed in Martinez *et al.* (2016) and will be discussed further below. The source of contaminating microbial species may be from the skin, oral cavity and/ or the implanted devices themselves. Once an infection is detected, the devices usually have to be replaced.

1.2 Tracheoesophageal Speech Valve

Laryngeal cancer patients who are treated by laryngectomy will experience loss of voice, impairment of gustation and will need to undergo voice rehabilitation to improve their quality of life. Laryngectomy is a surgical removal of the larynx due to cancer and a connection of the trachea to the neck through a circular opening called a tracheostoma is formed to enable breathing directly into the lungs, and bypassing the throat/mouth. The esophagus remains intact, allowing normal swallowing behaviour. The patient who undergoes this procedure is commonly known as a 'neck

breather'. In order to re-establish speech to these patients after laryngectomy, a surgical procedure, tracheoesophageal puncture (TEP) is done. The TEP method was initially published in 1979 and has subsequently been widely adopted (Brettholle, 1982). TEP involves the placement of a silicone tracheoesophageal speech valve (TESV) between the trachea and esophagus in order to restore voice function. The implantation of TESV pioneered by Blom and Singer has typically enjoyed success rates of about 68.7% to restore speech function while the rest failed to achieve phonation at the first attempt (Cruz *et al.*, 2014).

Currently, there are two types of TESV in the market; indwelling and non-indwelling devices. The device is not a permanent implant and needs to be replaced regularly, either by the patient (non-indwelling TESV) or by a clinician (indwelling TESV). The commonly used indwelling devices (manufactured by Groningen, Provox and Blom-Singer) contain a low-resistance valve and have a longer lifespan (Balm *et al.*, 2011). Patients attending the Freeman Hospital, Newcastle upon Tyne, use both indwelling and non-indwelling TESVs purchased from Blom-Singer (Owen and Paleri, 2013).

The function of the valve is to facilitate patients to produce sound via the exhalation of air from the lung. The TESV diverts the air in the trachea into the throat and mouth via a 1-way valve to enable voice-sound production with the tongue and lips. Voice production occurs by temporarily blocking the tracheostoma, so that exhaled air from the lungs can be directed from the trachea through the prosthesis into the esophagus, (where vibrations are produced), and then out through the mouth (refer to Figure 1-1). The TESV also acts as barrier between the esophageal area, which is exposed to food particles and fluid, to the trachea that connects to lower respiratory tract. As a one-way valve, the flow of air is directed from the trachea to the esophagus thus enabling restoration of speech function and at the same time preventing leakage of saliva or food into the airway from the newly 'reorganized' space after major throat surgery.
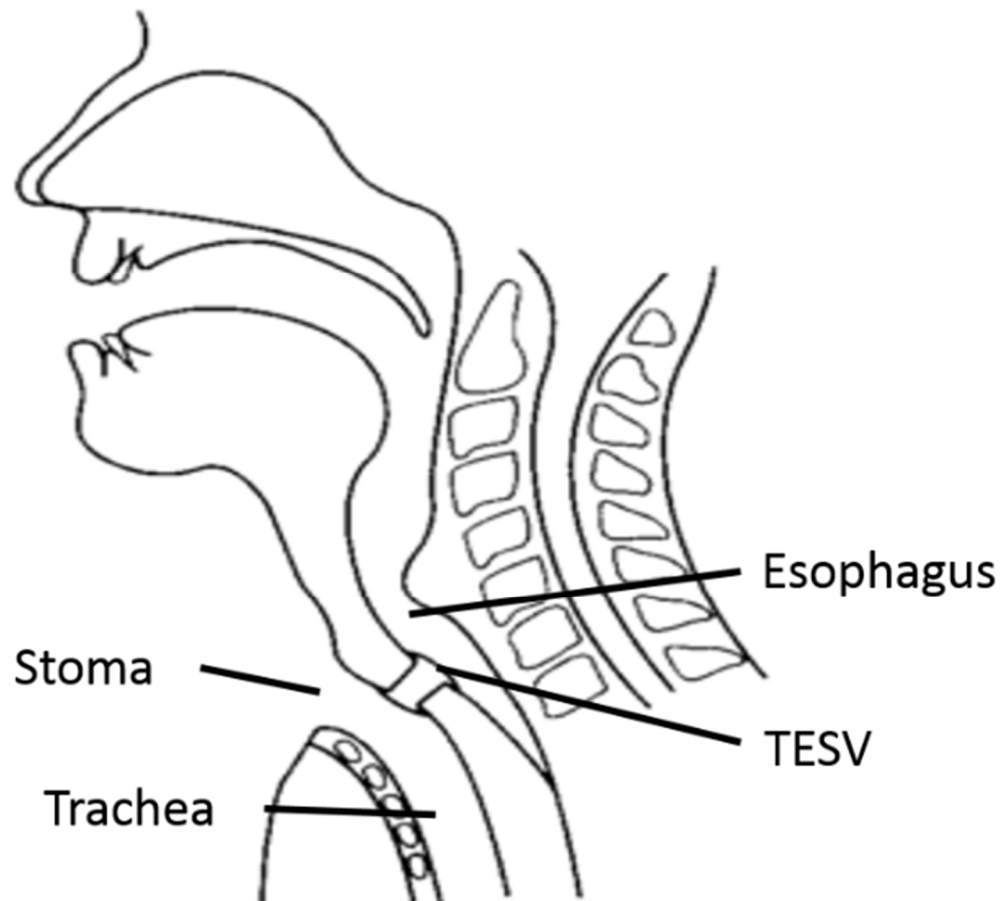
Figure 1-1: **The anatomy of laryngectomy.**
The tracheoesophageal valve prosthesis is placed in between the esophageal area, which is usually exposed to the food particles and fluid, and the trachea that connects to lower respiratory tract. During exhalation, when the stoma is closed to channel the air from the lung into the TESV, voice is produced via formation of vibration. The valve in the indwelling device will block the leakage of food or other substances from the esophagus into the trachea.

1.2.1 Problems faced by the TESV wearer

Factors that influence the formation of medically relevant biofilms include the types of microbial species present, atmospheric conditions (Cramer, 2014), nutrient supply and conditioning film. Changes in environmental pH and chemical composition may also influence the microbial composition of biofilms. Cells growing in biofilms are known to be phenotypically different from those growing planktonically as they exhibit different patterns of protein expression (Costerton *et al.*, 2003).

The position of biofilms on defective valves tends to be largely on the esophageal surface (Elving *et al.*, 2003). The esophagus is a part of the digestive tract and is lined with non-keratinized stratified squamous epithelium. This structure receives a regular supply of food particles and fluid as it connects the stomach with the oral cavity. The valve is supplied with saliva containing microorganisms such as *Staphylococcus aureus*, and this species has been isolated in high numbers from TESVs (Palmer *et al.*, 1993). Furthermore, the presence of saliva provides a nutrient rich environment, supporting the formation of a mixed species biofilm (Millsap *et al.*, 2001).

Most members of the microbial community in the biofilm that forms on TESVs can be considered opportunistic pathogens. Several studies have reported increased species richness in samples taken from immunocompromised patients (Neu *et al.*, 1994b; Bauters *et al.*, 2001; Honraet *et al.*, 2005). Patients that underwent radiotherapy treatment for larynx cancer presented with increased numbers of *C. albicans* (and other species) compared to patients who had cancer but not radiotherapy associated with the decreased salivary flow (Azizi and Rezaei, 2009). The surface properties of silicone are also important for microbial deposition. The hydrophobicity of silicone influences the formation of salivary conditioning films required for attachment (Busscher *et al.*, 1997).

The management of TESV is carried out by physical cleaning and also prophylactic antimicrobials. Currently, miconazole is the first choice for prophylactic treatment. The administration of antimycotic miconazole nitrate was reported to eliminate candida in the oropharynx of TESV wearers (van Weissenbruch *et al.*, 1997). The use of broad spectrum amphotericin B lozenges was also reported to be effective (Mahieu *et al.*, 1986) with a low incidence of resistance development (Torrado *et al.*,

2013). Although the administration of antimycotics can help to control *Candida* spp., the treatment is not effective for non-candida yeasts (van Weissenbruch *et al.*, 1997). Recent studies have also revealed that resistance to amphotericin B treatment is variable amongst *C. albicans* isolates as high biofilm formers (HBF) are more resistant than low biofilm formers (LBF) *C. albicans* (Sherry *et al.*, 2014). The prolonged use of some anti-mycosis agents also induces resistance to the drug (Denning, 1995). Cross-resistance is an additional potential problem. For example, Bauters *et al.* (2002) detected *C. albicans* isolates that were resistant not only to miconazole but also fluconazole. One of the properties of biofilm is increased antimicrobial resistance compared with planktonic cells, and this problem has been demonstrated in *C. albicans*. Thus the administration of antifungal amphotericin B at sufficient concentration to kill planktonic cells did not control *Candida* biofilms (LaFleur *et al.*, 2006). Valve cleaning is difficult due to the presence of biofilm. In order to facilitate cleaning, Shakir *et al.* (2012) reported that biofilm disruption is improved by the inclusion of a deoxyribonuclease.

1.3   Fungi in the Community: *Candida* spp.

Candida spp. are eukaryotic microbes that are part of the normal flora of the skin and also colonise the oral cavity, gastrointestinal tract, vagina and urogenital tract. They are a common causative agent for nosocomial infections (Brandt, 2002) and are associated with medical device-related infections (Kojic and Darouiche, 2004; Talpaert *et al.*, 2014). Although relatively harmless under normal conditions for most individuals, *Candida* spp. have the potential to cause disease if the host immune system is compromised or depressed in some fashion (Kourkoumpetis *et al.*, 2011). For example, those suffering diabetes or infection with HIV are more likely to acquire yeast infections. The prolonged use of antibiotics may also promote *Candida* infections and may lead to invasion of the gastrointestinal mucosa. Overgrowth of *Candida* in humans is called candidiasis (commonly known as thrush), with *C. albicans* the most predominant species. Along with *C. tropicalis*, *C. parapsilosis* and *C. glabrata*, *C. albicans* is thought to be responsible for up to 90% of these types of infection in humans. *Candida* spp. are also responsible for about 90% of denture–related stomatitis cases (a form of oral candidiasis) (O'Donnell, 2016). Systemic *Candida* infections, for example in the bloodstream (candidemia) or major organs (invasive candidiasis), occur in immunocompromised hosts and can be extremely

serious with a mortality rate of over 40% (Diekema *et al.*, 2012). Estimates suggest there are 400,000 life-threatening systemic *Candida* infections each year worldwide (Brown *et al.*, 2012) and Rajendran *et al.* (2016) reported that *C. albicans* bloodstream infection is a significant risk factor for mortality in Scottish patients over the period 2012–2013.

1.3.1 *The main player:* Candida albicans

C. albicans is a common member of the microflora on human skin as the growth of the organism on skin is normally limited by dryness. However rapid growth is promoted on broken skin or moister, folded regions (Goering and Mims, 2008). It was estimated to be present in the gastrointestinal and genitourinary tracts in 40%-60% of healthy adults, but does not grow well outside the human body (Odds, 1988). Metabolically the organism is able to ferment a range of simple sugars including glucose, sucrose and maltose to acid end products but is unable to catabolise lactose, a property that can be used to differentiate it from other *Candida* species (Meyers, 1972). When grown at room temperature on appropriate agar in the laboratory, *C. albicans* produces large, round, white or cream coloured colonies.

C. albicans is labelled as a pleomorphic organism due to its ability to grow unicellularly, as a budding yeast, or in either filamentous pseudohyphal or hyphal forms (Sudbery *et al.*, 2004) (as shown in Figure 1-2). The pseudohyphae are morphologically distinct from hyphae as they present with constrictions at the point of septation and are somewhat wider than true hyphal forms (reviewed in Sudbery (2011)). This feature produces complex structured biofilms of multiple cell types (round, budding yeast-form cells; oval pseudohyphal cells; and elongated, cylindrical hyphal cells) encased in an extracellular matrix (Chandra *et al.*, 2001; Ramage *et al.*, 2005; Fox *et al.*, 2014).This shape switching ability is thought to promote virulence, as the *C. albicans* hyphal form appears to play a role in invasion of epithelial and endothelial cells. The hyphae are thought to invade and cause damage via the secretion of hydrolytic enzymes (Filler and Sheppard, 2006; Phan *et al.*, 2007; Dalle *et al.*, 2010; Zhu and Filler, 2010) and the cytolytic peptide toxin candidalysin (Moyes *et al.*, 2016). In addition, it seems that yeast cells are able to resist phagocytosis and escape from engulfing macrophages by changing to the hyphal form (Lorenz *et al.*, 2004).
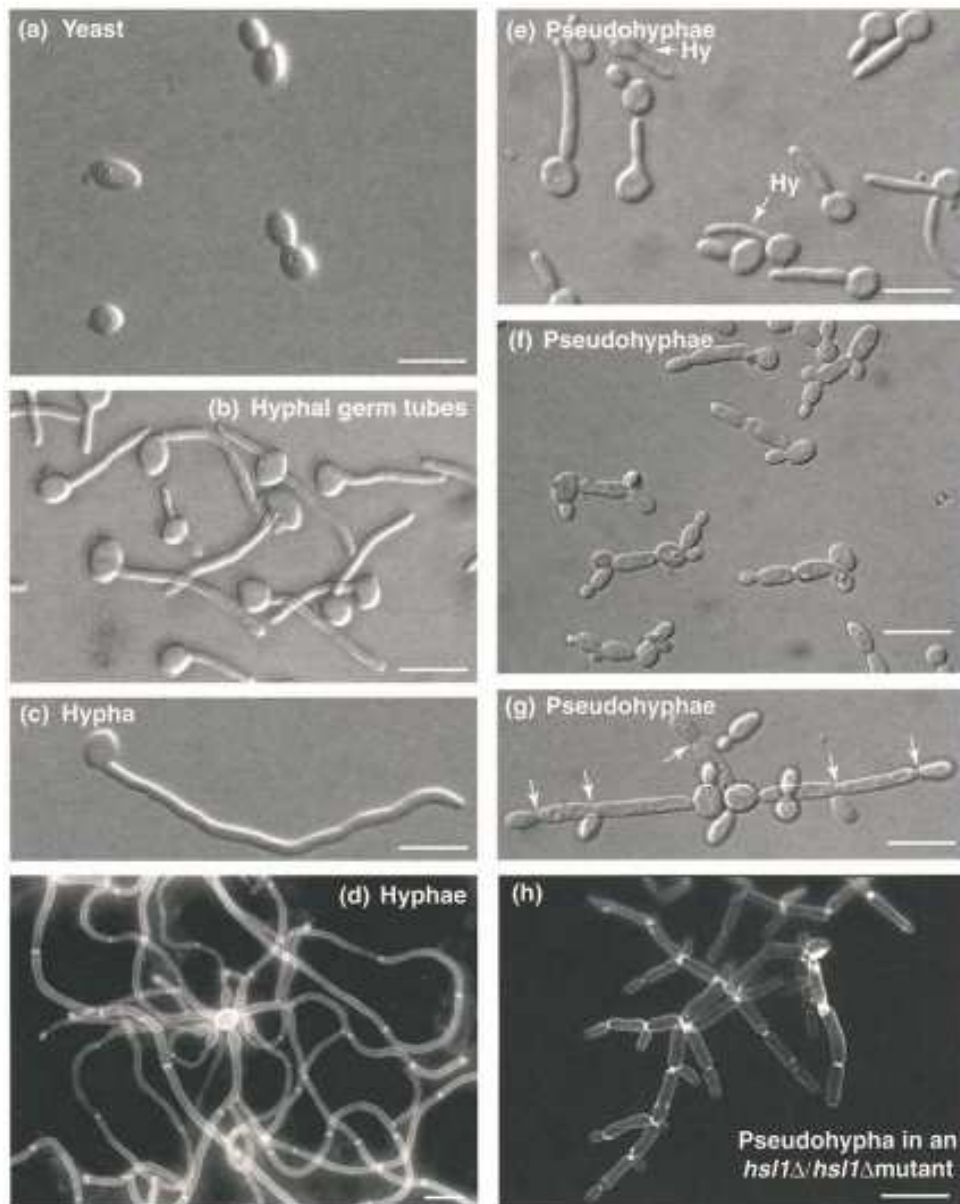
Figure 1-2: **Morphological states of *Candida albicans*.**
The cells initiate from (a) yeast budding cells, (b) hyphal germ tubes, (c,d) filamentous pseudohyphae to the formation of hypha (only one) or hyphae (multiple hypha). All the image was observed using DIC while the images in (d) and (h) are of cells stained with Calcofluor white, which stains chitin in the cell walls and septa and viewed using fluorescence microscopy. All scale bars represent 10 μm The micrograph is taken from Sudbery *et al.* (2004).

1.3.2 *Acquaintance:* **Candida glabrata**

Candida glabrata is a haploid member of the Saccharomycetaceae family of yeasts (Fidel *et al.*, 1999) as shown in phylogenetic tree in Figure 1-3. This organism is frequently co-isolated with *C. albicans* as an opportunistic pathogen of oropharyngeal candidiasis (Tati *et al.*, 2016). Despite a large evolutionary distance and the presence of highly repetitive large tandem repeats in *C. glabrata* it shares unexpected striking similarities to *S. cerevisiae* (Descorps-Declère *et al.*, 2015). The species used to be classified in the genus *Torulopsis* due to its lack of pseudohyphal formation. Due to its association with human infection, in 1978 it was proposed to reclassify *Torulopsis glabrata* into the genus *Candida* (Fidel *et al.*, 1999). *C. glabrata* can only ferment glucose and trehalose. *C. glabrata* is not polymorphic and grows only as yeast cells (blastoconidia) that are smaller (1–4 µm) than the blastoconidia of *C. albicans* (4–6 µm) (reviewed in Silva *et al.* (2012)). *C. glabrata* also has higher resistance to oxidative stress when compared to *C. albicans* (Cuéllar-Cruz *et al.*, 2008). According to a European Confederation of Medical Mycology, the incidence rates of candidosis infections caused by *C. glabrata* and *C. parapsilosis* was 14% (Tortorano *et al.*, 2006). *C. glabrata* infection is commonly found in cancer patients, denture-wearers, in those following prolonged use of broad spectrum antibiotics, steroids or undergoing head and neck radiation therapy (Silva *et al.*, 2012).
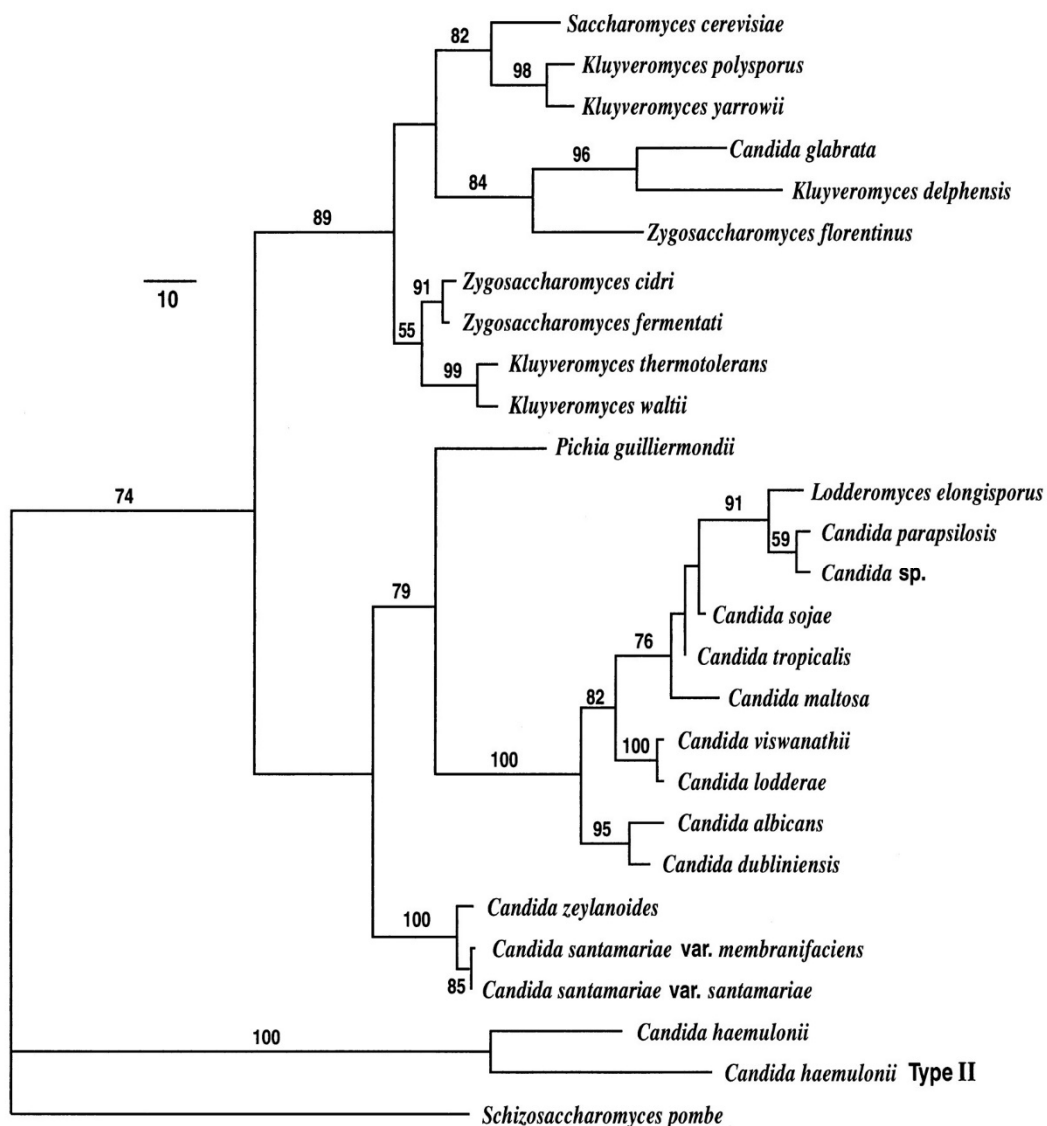
Figure 1-3: **Phylogenetic tree of the *Candida* species studied, and their nearest neighbours as represented by one of two most parsimonious trees derived from maximum-parsimony analysis (taken from Mannarelli and Kurtzman (1998)).**

1.4   Overview of Biofilms

Biofilms are ubiquitous in nature and consist of a consortium of microorganisms covering a surface and embedded in a matrix of extracellular polymeric substances (Costerton *et al.*, 1999). A new definition for medically relevant biofilms was introduced by Parsek and Singh (2003), who proposed specific criteria for medically relevant infections to be considered biofilms. These criteria include a requirement to demonstrate that infection caused by the biofilm is limited to a particular location and that the biofilm cells have enhanced resistance to antimicrobial agents. This is different from previous definitions because it is based on function (ie antimicrobial resistance) rather than just on structure.

Surfaces in humans are normally coated by a conditioning film, the nature of which depends on where in the body the material is placed, but it will largely be comprised of proteins or glycoproteins of host origin. Biofilm formation naturally occurs on all environmentally exposed surfaces that are moist and accessible to microorganisms. Biofilms can also be involved in diseases: for example, the development of dental plaque can lead to dental caries (Cortés *et al.*, 2011; Jakubovics, 2015). The National Institutes of Health has estimated that 80% of human infections result from pathogenic biofilms (http://grants.nih.gov/grants/guide/pa-files/PA-99-084.html).

Biofilms are structured communities of microbes, the final product of numerous synergistic and antagonistic reactions between the member community (Jakubovics and Kolenbrander, 2010). The biofilm architecture is largely determined by environmental conditions and polymicrobial interactions between cell surface adhesins and receptors that facilitate the adherence of many bacteria and fungi (Rickard *et al.*, 2003; Katharios-Lanwermeyer *et al.*, 2014). Various physical and chemical interactions between different microorganisms and their environment contribute to the development of community complexity. At least one member of any biofilm community contributes to the structure by producing some type of extracellular polymeric substance (EPS). The biofilm structure provides protection from external stresses, desiccation, and antimicrobial substances to the microbial community. In many cases, there are early colonizers that are specifically adapted to attach to the conditioning layer, and these modify the environment enabling other later colonizers to integrate as the mature biofilm develops (Diaz *et al.*, 2012a). Interestingly, Hope *et al.* (2002) showed that the upper layers of an oral biofilm

grown on hydroxyapatite (HA) discs contain proportionally more viable bacteria than the lower regions.

1.4.1 Stages in biofilm formation

Generally, stages in the biofilm development are attachment, maturation and stabilization and dispersion (reviewed in (Stoodley *et al.*, 2000); Gupta *et al.* (2016)). In the first stage, planktonic cells become reversibly adhered to a surface covered by a conditioning film. Attraction of microbial cells is influenced by the chemical nature of the surface of the cells and substratum (Bos *et al.*, 1999; Tribedi and Sil, 2014). Weak forces facilitate the primary associations. Hydrophobic interactions form between hydrophobic non-polar regions of the surface and the bacteria, reducing repulsive forces (Tribedi and Sil, 2014). The attractions are also facilitated by Van der Waals and electrostatic forces (reviewed in Garrett *et al.* (2008)). The initial stage is dependent on the ability of microorganisms to reach the surface, and can be influenced by fluid flow or active motility (Jakubovics and Kolenbrander, 2010). Cells remain immobilized and some become irreversibly attached by stronger specific adhesin/ receptor interactions to a surface between microbial cells and molecules in the conditioning film when the attractive forces are greater than repulsive forces (Stoodley *et al.*, 2000).

Once the initial adhesion is established, communication between sessile microbial cells increases, forming aggregates and microcolonies and the biofilm develops structural rigidity and protection from the external environment. The biofilm becomes multi-layered and the thickness gradually increases as the biofilm grows. The microcolonies consist of broad microbial communities and their size increases and, depending on the biofilm, the thickness may reach 100 μm or more. Substrate exchange occurs; distribution of metabolic products and removal of toxic end products are influenced by their proximity (Davey and O'toole, 2000).

During biofilm maturation the cells are encased within a self-produced matrix of extracellular polymeric substances (EPS) that is commonly comprised of lipids, proteins (including those that exhibit amyloid-like properties), extracellular DNA (eDNA) and exopolysaccharides (reviewed in Donlan and Costerton (2002); Hall-Stoodley *et al.* (2004)). The eDNA component of the matrix of *Enterococcus faecalis* biofilms has been described as 'yarn-like and sweater structures' when visualised by

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scanning electron microscopy (Barnes *et al.*, 2012). Similar structures have been identified and visualized using high-resolution field emission scanning electron microscopy in the polymicrobial matrices of natural subgingival dental plaque biofilms (Holliday *et al.*, 2015; Rostami *et al.*, 2017).

In the final stage of biofilm maturation, a point is reached where there is continual turnover of the cells, and cells in the outer layer are removed by passive or active processes. In some species, the sessile cells transition back to motility for dispersion (Hall-Stoodley *et al.*, 2004). In the case of *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* biofilms, Kaplan *et al.* (2003) reported that there is constant cell release (erosion), sloughing of sessile cells from the exterior of the biofilm, and seeding from internal cavities of the matured biofilm. Under certain conditions cells can detach from the biofilm structure and establish new biofilms on uncolonized surfaces. Detachment may occur via external mechanisms for example shear from increased fluid flow (Stoodley *et al.*, 2002a) or via internal processes such as enzymatic degradation of extracellular polymers or binding proteins (Kaplan *et al.*, 2003). This is important for the survival of the species in biofilms as it allows cells to escape competitors or harsh environmental conditions and to invade new environments. However, the biofilm structure itself provides a certain level of protection from external stress, desiccation and antimicrobial substances to the microbial community.

1.4.1.1 *Physical interaction: Coaggregation*

Microbial cells may initially associate through a binding mediated via hydrophobic interactions and/or electrostatic forces regardless of specific interacting surface macromolecules. However, due to their non-specific and relatively weak nature are not considered true coaggregation interactions (Katharios-Lanwermeyer *et al.*, 2014). Coaggregation occurs when genetically distinct microorganisms attach to one another via specific cell-surface adhesins and receptors (Ellen and Balcerzak-Raczkowski, 1977; O'Sullivan *et al.*, 2000), while auto-aggregation is the adherence of genetically identical bacteria to one another (Elliott *et al.*, 2006; Khemaleelakul *et al.*, 2006). Coaggregation between cells of differing species is thought to play a key role for multi-species biofilm formation. Specific binding between cells from different species may occur when planktonic cells from two species coaggregate and then attach to an existing biofilm. Alternatively free-floating cells from a species may

coaggregate with cells of a member species of an existing biofilm community, in a process known as 'co-adhesion' (Rickard *et al.*, 2003; Kolenbrander *et al.*, 2010). Eventually an organized structure forms that facilitates biofilm persistence and provides increased protection for member cells. The biofilm community develops into tangled cellular patchwork that facilitates cell-cell signalling and promotes metabolic cross-feeding between species (Davey and O'toole, 2000; Kolenbrander *et al.*, 2010). For an example of nutrient complementation, Zhou *et al.* (2016) reported that monocultures of the oral bacterial species *A. oris* and *S. oralis* grew poorly if at all when cultured in a saliva based medium, but grew strongly as coaggregates in the same medium in co-culture.

The specificity of adhesion facilitates coaggregation interactions which contribute to the microbial succession that occurs during biofilm development (Hojo *et al.*, 2009). In addition to being important in biofilm formation (Rickard *et al.*, 2003), these binding interactions are considered to be a virulence factor (Sundqvist, 1992) as they have potential to change the normal biofilm community by allowing colonization of more pathogenic microbes. It has been proposed that coaggregation of periodontopathogens in dental plaque contributes to the progression to periodontal disease from the healthy gum (Jakubovics and Kolenbrander, 2010). Coaggregation has also been shown to occur between species isolated from distinct and differing environments. The authors of this study proposed that these organisms be designated as 'cross-environment coaggregating organisms' (Stevens *et al.*, 2015). Thus, coaggregation also provides a mechanism that potentially can promote the translocation of microorganisms to new environmental niches.

The macromolecules that participate in coaggregation reactions can be either proteins (designated as adhesins) or polysaccharides (designated as receptors) (Kolenbrander and Phucas, 1984). Coaggregation reactions are specific and can form between adhesins and receptors (Kolenbrander *et al.*, 2006; Kline *et al.*, 2009) or two adhesins (Daep *et al.*, 2008). Although normally unimodal, occasionally the interaction can be bimodal where two different interacting pairs of macromolecules are involved in the coaggregation (Kolenbrander, 1982). One can determine which cell possesses an adhesin or receptor by exposure of each species to heat or protease. Whichever coaggregation partner possesses the adhesin or receptor can then be determined by assessing the effect of the treatment using a visual

coaggregation assay, a semi-quantified modified visual scoring method introduced by Cisar *et al.* (1979). There are a number of more quantitative methods available, but currently all have limitations and it is not clear that they are significantly better than the visual assay. For example, a high-throughput microplate-based approach was developed that enables rapid detection of coaggregation between candidate coaggregating pairs of strains simultaneously while controlling for variation between replicates (Levin-Sparenberg *et al.*, 2016). A FlowCam™ approach, involving the flow of particles past a high speed camera combined with rapid image analysis, allows for in-depth analysis of the rates of coaggregation and size of aggregates formed (Levin-Sparenberg *et al.*, 2016). Another method that has been employed to quantify coaggregation involves using spectrophotometry to monitor the reduction in optical density as large aggregates settle to the bottom of a tube. This method is however inappropriate for screening larger numbers of samples and it is not clear how well reduction in optical density correlates with coaggregate size (Ledder *et al.*, 2008; Arzmi *et al.*, 2015). Therefore the semi-quantitative visual assay has been used extensively (Kolenbrander *et al.*, 1995; Jakubovics *et al.*, 2008a; Silverman *et al.*, 2010; Vornhagen *et al.*, 2013; Sato and Nakazawa, 2014; Stevens *et al.*, 2015) in order to offer a simple reproducible, rapid and less technical method (Bos *et al.*, 1999) for broad screening of potential coaggregation pairs.

Further information regarding the chemical nature of the adhesin–receptor interaction can be obtained by assessing the ability of specific sugars or amino acids to either inhibit or reverse coaggregation (McIntire *et al.*, 1978). Using these relatively simple methods it was shown almost 40 years ago that coaggregation between, *Actinomyces viscosus* and *Streptococcus sanguis*, two-primary colonizing oral bacteria is mediated through surface-expressed protein adhesins and polysaccharide receptors, respectively (McIntire *et al.*, 1978). Also, the simple disaccharide lactose is known to inhibit some interactions between oral streptococci (Kolenbrander, 1988; Kolenbrander *et al.*, 2006). Other studies reported the effect of the amino acid L-arginine as an inhibitor of coaggregation between species (Kolenbrander *et al.*, 1995; Kolderman *et al.*, 2015). In addition, L-arginine was found to alter multi species oral biofilm development and composition, and caused a developed mixed species biofilm to disassemble. The proportion of *Streptococcus* spp. and *Veillonella* spp. increased and the proportion of Gram-negatives such as *Neisseria* and *Aggregatibacter* spp. was reduced in a controlled-flow microfluidic

system (Kolderman *et al.*, 2015). It was suggested that L-arginine might enhance the efficacy of antimicrobials to rapidly penetrate biofilms. However, in species like *Prevotella intermedia*, *Prevotella oris* and *Porphyromonas gingivalis* L-arginine inhibited coaggregation interactions (Sato and Nakazawa, 2014). *Fusobacterium nucleatum* has been reported to possess an outer membrane protein RadD that is responsible for arginine-inhibitable adherence (Kaplan *et al.*, 2008).

Although much of the research into coaggregation has investigated interactions between bacterial partners, *Candida* spp. also coaggregate with bacteria to anchor their position in the biofilm community. *S. gordonii* interacts with *Candida* spp. via adhesin-receptor on the *C. albicans* polysaccharide cell wall (Holmes *et al.*, 1996; Bamford *et al.*, 2009). Host proteins such as proline-rich proteins in saliva also assist the adherence of *C. albicans* to human surfaces such as tooth enamel hydroxyapatite (Cannon *et al.*, 1995; O'Sullivan *et al.*, 2000).

1.4.1.2   *Chemical/ molecular interaction: Quorum sensing*

Quorum-sensing (QS) (cell-to-cell communication) systems are employed by microbial species to promote collective behaviour within populations. In a biofilm environment, this phenomenon may provide advantages to the species such as resulting in increased access to nutrients or facilitating a defence against another competing species (Hogan, 2006; Nikolaev and Plakunov, 2007; Williams, 2007). Therefore, QS enables complex patterns of microbial group behaviour that arise from the coordinated activities of individual cells (Nikolaev and Plakunov, 2007; Williams, 2007). While the study of QS has been primarily undertaken for communication between cells within single species, it is now believed that these systems may be influenced by other species within a complex biofilm community. It appears now that the QS process spans the divide between prokaryotes and eukaryotes and that bacteria and fungi have developed signalling mechanisms to respond to each other. (Hogan and Kolter, 2002b; Joint *et al.*, 2002; Hogan, 2006; Williams, 2007). Although this area of research is still in its infancy, a number of interactions, particularly between *C. albicans* and oral streptococci have been reported (Hornby *et al.*, 2001; Bamford *et al.*, 2009; Wright *et al.*, 2013; Jack *et al.*, 2015).

For polymorphic fungi, it is probable that the net effect of both fungal and bacterial pro- and anti-filamentation signals determine the ratio of yeast and hyphal

morphotypes within biofilms. Various bacterial signalling molecules have been reported to influence the growth of *C. albicans*, hyphae formation, and biofilm development. Dual-species biofilms of *S. mutans* and *C. albicans* developed increased biomass and cell densities above those of single species. In addition, the *S. mutans* quorum sensing *sigX* system was stimulated resulting in promotion of important survival phenotypes (Sztajer *et al.*, 2014). In a similar *C. albicans*/ *S. gordonii* dual species biofilm model, the bacterial communication molecule *AI-2* (auto-inducer 2) increased biofilm formation and filamentation of *C. albicans* (Bamford *et al.*, 2009). Mixed biofilms that contained *C. albicans* and the *S. gordonii* Δ*luxS* mutant (unable to synthesise AI-2) produced significantly lower biomass and the number of yeast cells that produced hyphae were also lower (Bamford *et al.*, 2009). However, the *S. gordonii* competence stimulating peptide (CSP) appears to inhibit *C. albicans* biofilm formation but has no effect on the growth of hyphae (Jack *et al.*, 2015). Interestingly, the CSP produced by *S. mutans* appears to inhibit the formation of hyphae (Jarosz *et al.*, 2009; Vílchez *et al.*, 2010). Besides that, farnesol is an organic compound used by *C. albicans* as a quorum sensing molecule to inhibit hyphae formation (Hornby *et al.*, 2001). However, the effect is dampened in mixed biofilms with *S. gordonii* (Bamford *et al.*, 2009). Accordingly, an analysis of mRNA transcripts from mixed fungal and bacterial biofilms revealed an increased expression of *C. albicans* filamentation genes and those associated with protease and cell wall proteins compared with monoculture fungal biofilms (Dutton *et al.*, 2016).

1.4.1.3   *Synergistic and antagonistic interactions*

The biofilm composition and structure during development are strongly influenced by the interplay between a mixture of synergistic and antagonistic interactions between member species. Coaggregation, as well as providing the physical connections necessary for biofilm establishment, also facilitates various chemical interactions between partner cells by securing them in close proximity. These interactions include nutritional co-operation between species, gene transfer and cell–cell signalling. For bacterial biofilms, particularly dental plaque this topic has been extensively researched and reviewed (Hojo *et al.*, 2009; Jakubovics and Kolenbrander, 2010; Jakubovics, 2015).

There is evidence that interactions occur between different genera of commensal bacteria and *Candida* spp., and that these help to control overgrowth of *C. albicans* in the human oral cavity, gastrointestinal tract and female urinogenital tract (Falagas *et al.*, 2006; Neville *et al.*, 2015; O'Donnell *et al.*, 2015). Synergistic interactions between commensal bacteria and *Candida* spp. have also been observed (Xu *et al.*, 2016). The balance of these positive and negative influences determines the final compositional membership of the community. Understanding the essential features of these interactions will provide valuable knowledge required for prevention, control and treatment of mixed *Candida-* bacterial infections.

1.5   *Candida* spp. in Biofilms

Biofilm formation by *Candida* spp. in a model system is initiated by the attachment of yeast cells to the device surface. The colonization is continued by the proliferation of the cells and finally by the establishment of pseudohyphae and hyphae with the presence of an extracellular matrix (Hawser *et al.*, 1998; Ramage *et al.*, 2002a; Douglas, 2003).

1.5.1   *Candida albicans in biofilms*

Biofilms containing *C. albicans* usually containing various morphotypes (round yeast-form cells; ovoid pseudohyphal cells and elongated hyphal cells (shown in Figure 1-2) (Nobile and Johnson, 2015). As with all biofilms, the cell types are encased in an extracellular polymeric matrix and the biofilm architecture generally presents with yeast cells dominating the basal region and with hyphae surrounded by EPS presenting throughout (O'Toole *et al.*, 2000; Stoodley *et al.*, 2002b; Cramer, 2014). This structure largely reflects the various stages of biofilm development (a) initial surface adherence by yeast cells), (b) yeast cell proliferation to form an anchoring basal surface layer, (c) the triggering of pseudohyphae and eventually hyphae along with continued production of EPS material (O'Toole *et al.*, 2000; Stoodley *et al.*, 2002b; Ramage *et al.*, 2005).

In the clinical setting, clinical isolates of *C. albicans* showed some heterogenicity in forming biofilm, and were categorized into LBF and BHF (Sherry *et al.*, 2014). LBF isolates were reported as comprising a predominance of yeast cells and lack of hyphal cells, while *C. albicans* HBF appeared highly filamentous with a multi-

dimensional structure and very few yeast cells. It is important to identify this feature as (Sherry *et al.*, 2014) and (Hasan *et al.*, 2009) showed that HBF isolates caused significantly greater mortality rates than LBF isolates. The heterogenicity was regulated at various transcriptional levels and the expression of genes including those related to adhesion (*ALS3* (Bamford *et al.*, 2015), *EAP1* (Li *et al.*, 2007)), filamentation (*EFG1* (Ramage *et al.*, 2002b)) and resistance (*ZAP1* (Nobile *et al.*, 2009)) appear involved. Recent studies have confirmed that regulation of the *C. albicans* biofilm process is achieved by a complex integrated network of multiple transcription factors (Nobile *et al.*, 2012; Glazier *et al.*, 2017).

1.5.2 *Candida glabrata in biofilms*

C. glabrata, does not undergo the yeast-to-hyphae transition in contrast to *C. albicans.* The ability of *C. glabrata* to form biofilms in rich culture media *in vitro* was reportedly low compared to other non-*albicans Candida* (NAC) (Shin *et al.*, 2002; Silva *et al.*, 2009b). However, Silva *et al.* (2010) reported that *C. glabrata* produced a higher biofilm biomass on silicone surfaces in the presence of urine, compared with *C. parapsilosis* and *C. tropicalis. C. glabrata* forms biofilms various biomaterials, including cardiac devices and catheters (Kojic and Darouiche, 2004). Attachment and biofilm formation appear to be dependent a group of adhesins belong to the *EPA* and *AWP* gene families (Cormack *et al.*, 1999; Gallegos-García *et al.*, 2012; de Groot *et al.*, 2013). Specific *EPA* member's expression are up-regulated during adhesion to epithelial cells (Domergue *et al.*, 2005) and others during biofilm development (Kucharíková *et al.*, 2011). Members of the *EPA* family that are involved in biofilm development are tightly regulated and dependent on environmental conditions and growth stage (Kraneveld *et al.*, 2011)((Iraqui *et al.*, 2005). Although lacking the yeast-to-hyphae transition observed in *C. albicans*, *C. glabrata* is able to form biofilms consisting of yeast cells encased in EPS (Seneviratne *et al.*, 2009) consisting of proteins and carbohydrates (Silva *et al.*, 2009a). The ability to form a biofilm via adherence to either a host surface or abiotic medical device is an important virulence factor.

Similar to *C. albicans*, *C. glabrata* carries one catalase gene, *Cat*1, and a single AP-1-like transcription factor, *Yap*1, that regulates the induction of genes such as *Cat*1 which convert H_2O_2 to H_2O and molecular oxygen. The oxidative stress responsive

transcription factor, *Yap*1, is also involved in resistance to different drugs (Chen *et al.*, 2007) enabling the NAC possesses high level of resistance to several antifungal drugs (González *et al.*, 2008). In clinical settings, Azoles such as fluconazole and itraconazole are the most frequently used antifungals that have a fungistatic activity against *Candida* spp. However, *C. glabrata* is highly resistant to such azoles (reviewed in (Silva *et al.*, 2012)).

1.6   *Candida albicans* in Mixed Species Biofilms

C. albicans is often found associated with bacteria in mixed infections. Biofilms containing both *C. albicans* and *Pseudomonas aeruginosa* regularly form in patients with indwelling catheters, lung infections, cystic fibrosis sufferers, and those who develop infections following burns (reviewed by De Sordi and Mühlschlegel (2009)). The various synergistic and antagonistic interactions of the community members largely determine the architecture of the resulting biofilms. For example, *C. albicans* provides a hypoxic microenvironment in mixed biofilms cultured in ambient oxic conditions that supports the growth anaerobic bacteria (Fox *et al.*, 2014) (as shown in Figure 1-4). Cramer (2014) demonstrated that *C. albicans* metabolism lowering of oxygen tension facilitates growth of anaerobic bacteria promoting development of a dual-species biofilm.
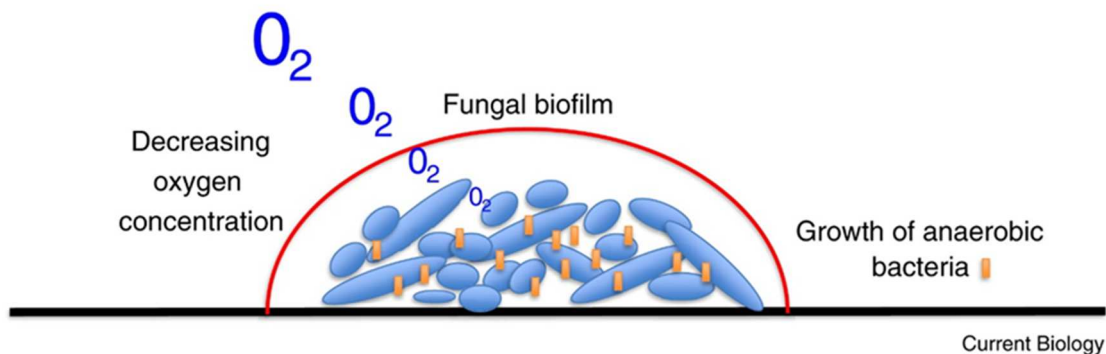
Figure 1-4: Fungal biofilms structure with the presence of bacteria species. The biofilm is heterogeneous with multiple morphological forms present including yeast (blue round ovals), pseudohyphae (slightly elongated blue ovals), and hyphae (fully elongated hyphae). The low oxygen (hypoxic) regions of biofilm supports anaerobic bacterial growth. surrounding the biofilm is an extracellular matrix (red outline, white interior) largely composed of polysaccharides the oxygen gradients drop drastically, from the exterior of the biofilm colony to the interior near the surface (black), allowing proliferation and survival of anaerobic bacteria (orange rods) (modified from Cramer (2014)).

As mentioned previously the vast majority of microbial communities from failed TESVs biofilms are mixed predominantly containing commensal fungi and bacteria. Studies that investigate the structural architecture of mixed fungal/bacterial biofilms are relatively rare. However, in (2016), Cavalcanti and colleagues developed an *in vitro* model to determine the effect of interactions of two early-colonizing denture plaque bacterial species, *Streptococcus oralis* and *Actinomyces oris* on the incorporation *C. albicans* into the developing biofilm. *C. albicans* hyphae were observed in the resulting multi-species biofilms to be incorporated throughout the biofilm layers.

In fungal/bacterial dual-species biofilms, *S. oralis* developed a basal layer of cells after co-cultured with *C. albicans* at the same time. The bacterial cells appeared to colonize areas unoccupied by *C. albicans* on the salivary pellicle. Streptococci were observed in patches throughout the biofilm at 24 hours (Cavalcanti *et al.*, 2016). This finding was in agreement with that reported by Bertolini *et al.* (2015) for similar dual-species biofilms. *A. oris,* by contrast, was predominantly associated with *C. albicans* hyphal filaments, failing to substantially colonize the pellicle in 24-hour biofilms. Synergistic interactions were observed with viable bacterial numbers higher in biofilms containing *C. albicans* than in its absence.

A study by van der Mei *et al.* (2014) also used a similar dual species *in vitro* model system employing silicone rubber. They studied biofilm formation on the silicone surface by *C. albicans* (or *C. tropicalis*) along with a variety of commensal bacterial species isolated from failed TESVs including *Lactobacillus* strains. Interestingly, they found there were significantly fewer viable organisms in biofilms containing *C. albicans* and a bacterial strain compared to the equivalent biofilms containing *C. tropicalis*. Biofilms grown with mixtures of *Candida* spp. and lactobacilli contained high proportions of yeast. *L. casei*, reduced *Candida* hyphal formation in biofilms compared to those grown in absence of bacteria or grown in the presence of *Rothia dentocariosa*. Previous studies have suggested that lactobacilli are associated with voice prostheses with extended clinical life-times and *Rothia* spp. with short clinical life-times (Elving *et al.*, 2002; Buijssen, 2012).

Buijssen *et al.* (2007) employed polymerase chain reaction, denaturing gradient gel electrophoresis and fluorescence in situ hybridization to investigate the microbial composition of biofilms from 33 explanted TESVs. In this study lactobacilli were

shown to be regular and significant colonizers of TESVs for the first time. These bacteria were found to be closely associated with the *Candida* species in the biofilm. Five years later, the same group studied the biofilm architecture of 22 failed silicone voice prostheses by employing FISH and CLSM (Buijssen *et al.*, 2012). There was wide variation in the prostheses lifetimes from just one week to more than four years. They observed that generally the biofilm was cross-sectionally thicker with age. They found that the bacterial cells were usually found close to the outermost surface of the biofilm whereas yeasts were clustered in bags of colonies near to the surface or embedded in the silicone rubber. Yeasts growing in the bags failed to form hyphae. Hyphae were predominantly observed in the extremities of the biofilm around the silicone surface. Extracellular polymeric substance was usually more prominent in older biofilms. In addition, specific FITC-labelled probes revealed that although *C. albicans* was detected in most prostheses other fungal species were present.

1.6.1 *Environmental factors that induce hyphal growth in* C. albicans*.*

As noted in Section 1.5.1, the yeast-hyphal transition is thought to be a key factor in biofilm formation by *C. albicans*. Hyphal growth by *C. albicans* can be classified into two distinct stages. The first stage is the initiation of hyphal growth and its short-term maintenance and is assessed over a period of hours and the second stage is the maintenance of hyphal growth in the longer term. Hyphal formation occurs in response to several environmental conditions that reflect the environment encountered in the host *in vivo*.

The initiation of hyphal growth can be triggered by temperatures of 37°C and exposure to serum, which rapidly induces germ tube formation. In fact, this observation is routinely used as the standard laboratory method for identifying *C. albicans*. The test requires induction of hyphae when cultured in horse serum at 37°C for up to 4 hours. The morphological switching in *Candida* cells illustrated in Figure 1-2 is influenced by different environmental signals. For example, high temperature, high ratio of CO_2 to O_2, neutral pH, and nutrient poor media stimulate hyphal growth and restrict yeast cell growth (Sudbery, 2011). The switching between yeast and hyphae is also stimulated by various chemical signals with other *C. albicans* cells or those of different microbial species. This microbial communication system, known as quorum sensing (discussed previously in section

1.4.1.2), enables the *Candida* to sense the numbers other similar cells in the immediate environment via secretion of the QS molecules. The main QS molecules in *C. albicans* are farnesol (Ramage *et al.*, 2002a) and aromatic alcohol tyrosol (Chen *et al.*, 2004). Farnesol has been studied as a hyphal formation inhibitor and also as a mechanism to promote resistance to oxidative stress (Oh *et al.*, 2001; Ramage *et al.*, 2002a). In mixed communities detection of farnesol in sufficient quantity indicates a dominance of yeast cells in the community and inhibits hyphal switching (Kruppa, 2009).

The second stage of the switching mechanism is the maintenance of hyphal growth in the longer term. In this stage the ability to form hyphae can be assessed either by their formation in liquid culture or by the appearance of hyphal colonies when grown on a solid medium. It is determined by assessing colonial morphology following 5 days' growth at 37 °C on agar medium containing serum. Hyphal formation on solid medium results in the main colony changing from a smooth to a crumpled appearance and the development of spidery outgrowths with mature hyphal colonies often invading the agar (reviewed by Sudbery (2011)).

However with the presence of bacteria, filamentation in fungal can be inhibited often by secreted QS compounds, virulence factors, or metabolism (Morales *et al.*, 2013; Matsubara *et al.*, 2016). In these biofilms the *P. aeruginosa* cells exclusively bind to the germ tubes of *C. albicans* which leads to the death of the germ tube. In addition, *Candida* cells sense secretion of 3-oxo-homoserine lactone by the bacterial cells, a chemical signal that acts to repress hyphae formation. Therefore, the presence of *P. aeruginosa* encourages the *Candida* sp. to maintain the yeast cell morphology by destroying hyphae and deterring their formation and increases the complexity of the infection system. (Hogan and Kolter, 2002a; Hogan, 2006).

1.6.2 *Adaptation to host environments*

One of the foci of the current study is to increase understanding of *C. albicans* stress signalling responses, particularly in relation to stresses produced by co-colonising bacteria. The capacity of *C. albicans* to survive in humans is largely due to its ability to quickly respond to host-generated environmental stresses as it predominantly found in mixed species biofilms in the host and thus needs to adapt to such environments (Brown *et al.*, 2014). Often the bacteria co-existing with *C. albicans* are

H_2O_2 producers so the ability to withstand oxidative stress may be important. The effects of different bacteria are discussed further in section 1.8.

In order to successfully colonize a host, *C. albicans* must effectively adapt to numerous local environmental stresses while responding to changes in carbon sources and/or fluctuations in temperature. The organism is regularly challenged by a variety of changes, such as alterations in nutrient supply, pH, temperature and exposure to oxidative and osmotic stresses (Setiadi et al., 2006; Pierce et al., 2013; Enjalbert et al., 2006; Lorenz et al., 2004). The stress resistance of *C. albicans* cells has been shown to be influenced by alterations in carbon source or temperature (Kastora *et al.*, 2017). For example, ambient temperature was shown to significantly affects the resistance of *C. albicans* cells to cell wall stresses, but not osmotic stress (e.g sodium chloride) (Leach *et al.*, 2012). Changes in exposure to carbon sources can strongly influence stress responses in the yeast. *C. albicans* oxidative stress resistance is enhanced in the presence of glucose (Rodaki *et al.*, 2009) whereas a switch from glucose to lactate as a carbon source conveys enhanced osmotic stress resistance (Ene *et al.*, 2012a; Ene *et al.*, 2012b).

1.7   Effects of environmental stress on *Candida* spp.

Microorganisms routinely generate reactive oxygen species (ROS) when molecular oxygen (O_2) is used for respiration and nutrient oxidation to generate energy. The formation of ROS or chemically reactive molecules is due to addition of electrons to molecular oxygen O_2, generating superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\bullet OH$), and singlet oxygen ($^1O_2$) (Gerschman *et al.*, 1954; Imlay, 2013). The superoxide becomes more toxic when it interacts with other O_2^- to form H_2O_2 or forms a very potent oxidant and reactive nitrogen species, peroxynitrite with nitric oxide (Pacher *et al.*, 2007). $^1O_2$ is a photoexcited form of O_2 and is particularly reactive with cysteine, histidine, methionine, tyrosine and tryptophan residues, unsaturated lipids and some nucleic acids (Briviba *et al.*, 1997). Even though H_2O_2 is relatively stable when compared to the other oxidants, in the presence of UV radiation, it becomes a precursor of free radicals. $\bullet OH$, the highly reactive but indiscriminate ROS is formed through the cleavage of the oxygen–oxygen bond of H_2O_2 and diffuses into cells and reacts with many biomolecules (Bokare and Choi, 2014). The accumulation of ROS within cells leads to a condition called oxidative stress (Cabiscol *et al.*, 2000; Green and Paget, 2004; Imlay, 2013), and ultimately

can result in protein, DNA, and lipid damage (Halliwell, 2006), and an increased rate of mutagenesis, leading to irreversible cellular damage or even cell death in *C. albicans* (Phillips *et al.*, 2003; Imlay, 2013).

In order to prevent the action of ROS, cells can deploy protective mechanisms such as the activities of detoxifying enzymes (e.g., superoxide dismutases and catalases). *Candida* spp. also alter their metabolism and their defence strategies to take advantage of the accumulated oxygen while avoiding the damage caused by ROS (Dantas *et al.*, 2015). Interestingly, in a biofilm, microorganisms are able to tolerate oxidative stress and use ROS as a signal to prepare for adaptation to a changing of environment (Imlay, 2013).

1.7.1 *Oxidative stress responses in* Candida albicans

C. albicans is more resistant to oxidative stress following exposure to H_2O_2 than other yeasts such as *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Nikolaou *et al.*, 2009) but not as resistant as *C. glabrata* (Cuéllar-Cruz *et al.*, 2008). Transcriptional responses to oxidative stress, involve a set of antioxidant genes that include catalase (*CAT1*) and superoxide dismutase (*SOD*) antioxidant-encoding genes (Enjalbert *et al.*, 2006). These are thought to be important virulence determinants in systemic models of infection.

To date, three signalling pathways have been demonstrated to be directly activated in response to ROS in *C. albicans* which include the *Cap*1 transcription factor, the Hog1 stress-activated protein kinase and the Rad53 DNA damage checkpoint kinase (reviewed in Dantas *et al.* (2015)). The role and regulation of Hog1 (high osmolarity glycerol response) stress-activated protein kinase in oxidative stress responses in *C. albicans* is discussed further in the next paragraph.

1.7.2 *Oxidative stress signaling / SAPK Hog1 pathway*

Oxidative stress is thought to be a major determinant leading to heterogeneity within biofilm communities (Saint-Ruf *et al.*, 2014). Each individual cell in a biofilm is exposed differentially to the surrounding environment, sense ROS at different levels, and activates its own ROS scavenging mechanisms, thus creating gradients of

different ROS forms and increasing the variety of phenotypes in the biofilm (reviewed by Gambino and Cappitelli (2016)).

The Hog1 (high osmolarity glycerol response) stress-activated protein kinase (SAPK) pathway in yeast cells was first described by Brewster *et al.* (1993). It is now established that all eukaryotes possess these important stress-signalling modules. The SAPK is a mitogen-activated protein kinase (MAPK) that promotes the ability of cells to adapt to environmental change (Smith *et al.*, 2010). The SAPK pathway consists of three-tiered core signalling module comprising of the SAPK, a MAP kinase kinase (MAPKK) and a MAPKK kinase (MAPKKK)). This kinase cascade activates the SAPK by phosphorylation of conserved residues on a TXY (threonine-x-tyrosine) motif. Once phosphorylated, the activated SAPK determines the appropriate response by in turn phosphorylating a number of target substrates in the cytoplasm and nucleus.

C. albicans Hog1 (*Ca*Hog1) is robustly phosphorylated and rapidly accumulates in the nucleus after the exposure of cells to a number of stresses including osmotic and oxidative stress (Smith *et al.*, 2004; Enjalbert *et al.*, 2006). In addition, it plays a role in morphogenetic regulation in repressing the yeast to hyphal transition under non-inducing conditions (Alonso-Monge *et al.*, 2003). Studies showed that *C. albicans* cells lacking Hog1 are more sensitive to oxidative and osmotic stress, demonstrating that Hog1 activation is a critical component of these stress responses in *C. albicans* (Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004). *Ca*Hog1, like *S. cerevisiae* Hog1, is regulated by a single upstream MAPKK Pbs2. CaPbs2 in turn is regulated by a single MAPKKK, CaSsk2 (Cheetham *et al.*, 2007), which contrasts with the Hog1 pathway in *Saccharomyces cerevisiae*, in which Pbs2 is regulated by three MAPKKKs (Ssk2, Ssk22, and Ste11) (Posas and Saito, 1997; Posas and Saito, 1998) as shown in Figure 1-5.
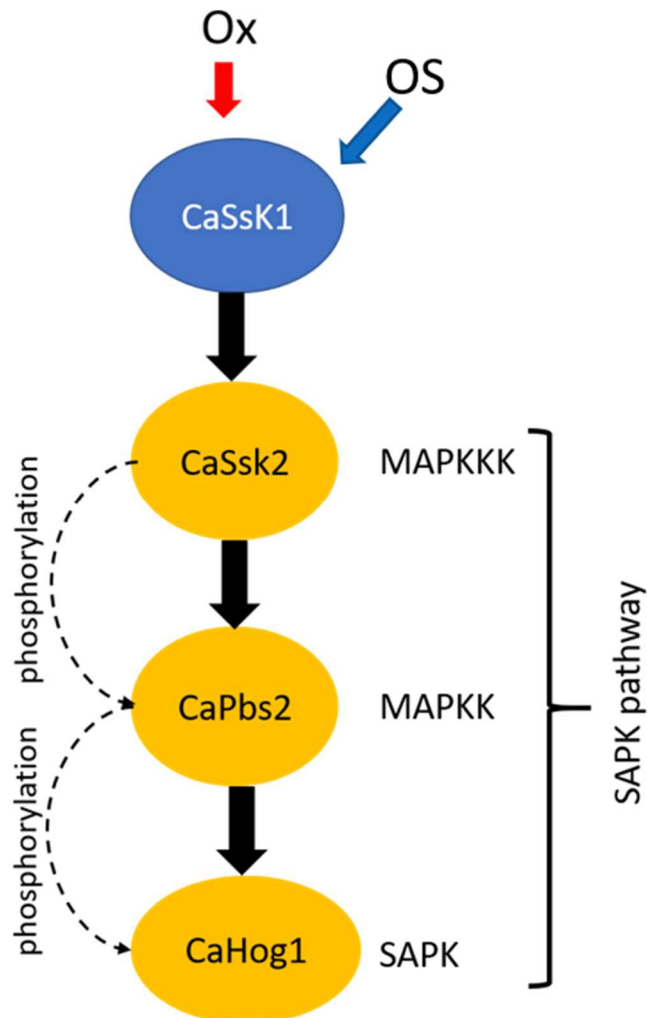
Figure 1-5: **Stress signalling to *C. albicans* SAPK pathways.**
The activation of the MAPKKK results in the phosphorylation and activation of the MAPKK, which, in turn, culminates in the phosphorylation of the SAPK. Ox, oxidative stress; OS, osmotic stress (modified from (Smith *et al.*, 2010).

1.8 Impact of bacteria on *Candida* in polymicrobial biofilms

Lactobacillus, *Streptococcus* and *Staphylococcus* have all been found in association with *Candida* on TESVs (Somogyi-Ganss *et al.*, 2017) and these genera are also commonly associated with *Candida* in other locations on the human body.

1.8.1 Lactobacillus *in polymicrobial biofilm*

Lactobacillus is a genus of Gram positive rod-shaped bacteria that comprise a significant component of the lactic acid bacteria group. Lactobacilli also produce bacteriocins and small anti-bacterial peptides (Messaoudi *et al.*, 2013; Pandey *et al.*, 2013). This genus is regularly isolated as part of the human microbiota at a number of body sites. Numerous studies have reported that *Lactobacillus* spp. have an inhibitory effect on *C. albicans* (Ceresa *et al.*, 2015; Parolin *et al.*, 2015; Rybalchenko *et al.*, 2015; Matsubara *et al.*, 2016). Although these studies used a variety of species, strains and methods, the overall conclusion was that lactobacilli adversely affect the colonisation and growth of *Candida* spp. Lactobacilli were shown to influence *Candida* in a variety of ways, including reduction of *Candida* attachment to host cells (Ceresa *et al.*, 2015), regulating hyphae formation (Liang *et al.*, 2016) and production of a cidal/static effect (Parolin *et al.*, 2015; Rybalchenko *et al.*, 2015).

Lactobacilli ferment sugars to lactic acid, thereby lowering the environmental pH thus creating a relatively acidic surrounding for those in the vicinity (Messaoudi *et al.*, 2013). This creates a selective advantage for any acidophilic organisms not only lactobacilli. *Candida* spp. are able to withstand these conditions, but they undergo a stress response that triggers hyphae formation. *L. casei* in particular is known to encourage hyphal growth in *C. albicans* (Orsi *et al.*, 2014). Interestingly, although it is known that some *Lactobacillus* spp. produce H_2O_2, one of the environmental stressors for *C. albicans*, the effect of it alone on *C. albicans* growth inhibition appears to be minor (Strus *et al.*, 2005; Kaewsrichan *et al.*, 2006; Parolin *et al.*, 2015). Lactobacilli are also known to produce other antimicrobials, biosurfactants and bacteriocins (Ceresa *et al.*, 2015). Biosurfactants are amphipathic molecules with detergent activity and those produced by a number of *Lactobacillus* spp. exhibit broad range cytotoxicity against Gram positive bacteria and *C. albicans* (Zakaria Gomaa, 2013). A biosurfactant produced by *L. brevis* significantly reduced the ability of *C. albicans* to bind to silicone surfaces and effectively slowed biofilm growth

(Ceresa *et al.*, 2015). However, Kaewsrichan *et al.* (2006) reported that H_2O_2-dependent activity by *L. jensenii* alone was insufficient to inhibit fungal growth. Matsubara *et al.* (2016) recently demonstrated the wide-ranging effects of three probiotic strains on *C. albicans*. They reported a negative effect of all three strains on the pathogenic switch from yeast to hyphae form. The presence of the strains also disrupted *Candida* spp. biofilms. The antagonistic effects are likely via both cell-cell interactions and exometabolites.

1.8.2 Streptococcus *spp. and their effects on* Candida

Some interactions between streptococci and *Candida* can be beneficial, others antagonistic (reviewed in Xu *et al.* (2014a)). It has been reported that streptococci can induce oxidative stress and hyphae production in *C. albicans* via the liberation of hydrogen peroxide (Jenkinson *et al.*, 1990; Nasution *et al.*, 2008). On the other hand, *C. albicans* co-aggregates with various oral streptococci, which may promote biofilm growth (Xu *et al.*, 2017). Oral streptococci are common "early colonisers" of both hard and soft tissue surfaces in the oral cavity (Diaz *et al.*, 2012a) and are also common members of TESV biofilm communities. Indeed, the majority of these are members of the "mitis group" of oral streptococci that includes *S. mitis*, *S. oralis*, *S. gordonii*, *S. sanguinis* and *S. parasanguinis* (Bryskier, 2002). Oral streptococci are frequently found closely associated with *C. albicans* in denture-associated stomatitis and diseases of the oral mucosa (Campos *et al.*, 2008). They can develop mutualistic relationships where the fungi promote streptococcal biofilm formation and pathogenicity (Falsetta *et al.*, 2014; Xu *et al.*, 2014b) and the bacteria appear to enhance the invasive properties of the fungus (Diaz *et al.*, 2012b; Xu *et al.*, 2014a; Palmer *et al.*, 2017).

Jenkinson *et al.* (1990) showed that *C. albicans* can bind directly to many of these species and streptococcal binding to both *C. albicans* morphotypes, yeast cell and hyphal forms has been microscopically observed (Dutton *et al.*, 2014). For example, the formation of dual-species *S. gordonii*-*C. albicans* biofilm communities involves an interaction of the *S. gordonii* SspB (adhesins cell surface protein) protein with the ALS3 (agglutinin-like sequence) protein on the hyphal filament surface of *C. albicans* (Holmes *et al.*, 1996; Dutton *et al.*, 2014).

Candida spp. provide adhesion sites (Holmes *et al.*, 2006; Nobbs and Jenkinson, 2015) and reduce oxygen tension levels, via respiration, to redox levels preferred by streptococci (Fox *et al.*; Cramer, 2014). For example, *S. oralis* showed the ability to form attachment on *C. albicans* germ tubes when in nutrient poor environments and was found to be attached to hyphae of *C. albicans* (Diaz *et al.*, 2012b) in nutrient rich media. The fungi may also provide stimulatory factors arising from nutrient metabolism that promote growth of the bacteria (O'Sullivan *et al.*, 2000; Jenkinson and Douglas, 2002). At the same time, the streptococci produce lactate which can serve a carbon source for growth for *Candida* spp. in addition to other nutrients that promote yeast growth (Brogden, 2002) These *Streptococcus* spp., similar to lactobacilli, are saccharolytic and therefore can assist, via carbohydrate fermentation, in creating an acidic environment, preferred by *Candida* spp. (Takahashi and Nyvad, 2011). The synergistic nature of the relationship between the two groups is further underlined by observations that *C. albicans* stimulates exopolysaccharide production by *S. mutans* when the two are grown together and that biofilms of the two species contain increased numbers of bacterial cells than mono-culture biofilms in the same system (Falsetta *et al.*, 2014). Falsetta *et al.* (2014) also showed that when both species were co-infected in a rat model of caries, biofilm virulence increased, and disease onset was more aggressive and severe than infection with either species alone. Similar effects were seen regarding the mucosal inflammatory response in the host, whereby streptococcal co-infection augmented the response to *Candida* (Xu *et al.*, 2014b; Xu *et al.*, 2017).

1.8.3 Staphylococcus *in polymicrobial biofilm*

C. albicans and *S. aureus* can also coexist within polymicrobial biofilms in humans (Shirtliff *et al.*, 2009). These species have received attention recently because of the increasing awareness of involvement of their biofilms in chronic and systemic infections. They are the most frequently isolated pathogens in bloodstream and systemic infections in patients requiring hospitalization (reviewed by Perlroth *et al.* (2007)). *S. aureus* is known to strongly attach to *C. albicans* hyphae, but does not bind strongly to the yeast form (Ovchinnikova *et al.*, 2012). This phenomenon has implications for pathogenesis as co-inoculation of these microbes leads to the development of a more severe infection than either alone. In addition, hyphae-bound *S. aureus* cells are more resistant to vancomycin treatment than monocultures

(Harriott and Noverr, 2009). El-Azizi *et al.* (2004) found that the adhesion of *C. albicans* to buccal mucosa was increased in the presence of staphylococcal proteinase. The physical relationship between *C. albicans* and *S. aureus* in mixed species biofilms, was initially shown with scanning electron microscopy (Costerton *et al.*, 1999; Adam *et al.*, 2002), and confirmed recently *in vitro* using fluorescence in situ hybridization (Shirtliff *et al.*, 2009).

A synergistic effect between *C. albicans* and *Staphylococcus* spp. was first described by Carlson (1983). In the study, *C. albicans* and *S. aureus* stimulated infection when co-inoculated intraperitoneally in mice and resulted in 100% mortality, while there was no fatality of any mice when species were administered separately (Carlson, 1983). It was proposed that the candidal infection process caused physical damage to organ walls by hyphae (Wilson *et al.*, 2016), facilitating penetration by other microbes . Proteomic analysis by Peters *et al.* (2010) revealed differential expression patterns in each species when grown in mixed biofilms compared with the equivalent monocultures. These findings demonstrated that, in addition to the induction of stress related proteins hyphae-binding of bacteria resulted in differential regulation of virulence factors such as upregulation of staphylococcal L-lactate dehydrogenase 1, which provides enhanced resistance to host-derived oxidative stressors. Therefore, increases in pathogenesis of *S. aureus* in mixed infection with *Candida* may be due to both physical interactions and increased expression of specific proteins associated with disease (Peters *et al.*, 2010). *S. epidermidis* also gained protection against vancomycin when present together with *C. albicans* and at the same time produced extracellular polymer that could inhibit fluconazole penetration in mixed fungal-bacterial biofilms (Adam *et al.*, 2002).

Although the majority of interactions between *C. albicans* and *Staphylococcus* spp*.* appear to be synergistic during biofilm development, some antagonistic interactions may arise. It has been shown that the candidal QS molecule farnesol can adversely affect *S. aureus* containing biofilm by disturbing cell membrane integrity, cell viability and increasing susceptibility to antibiotics (Jabra-Rizk *et al.*, 2006).

1.9   Common microbiome of TESV

There have been several studies of the microbiota in biofilms on TESVs and a number of common species have been identified (Mahieu *et al.*, 1986; Neu *et al.*, 1993; Elving *et al.*, 2002; Buijssen *et al.*, 2012). These communities are perhaps best characterised as mixed biofilms containing both commensal fungi and bacteria. *Candida* is commonly associated with these biofilms. *C. albicans* is reported as the predominant species, along with *C. tropicalis* and *C. glabrata* (Neu *et al.*, 1994b; Bauters *et al.*, 2002).

Studies showed that a number of oral bacteria also play important role in formation of TESV biofilm community including *Rothia denticariosa*, *Stomatococcus mucilaginosus*, *S. mitis*, *Streptococcus sobrinus* and *Streptococcus salivarius* (Neu *et al.*, 1994b; Somogyi-Ganss *et al.*, 2017). Also important are bacteria from the skin such as *Staphylococcus* spp. (Neu *et al.*, 1994b). There have also been reports of anaerobic and microaerophilic of oral pathogens such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Bertl *et al.*, 2012) as well as several opportunistic fungal pathogens that are present in the TESV of immunocompromised subjects such as *Fusarium solani* (Honraet *et al.*, 2005) and *Crytococcus neoformans* (Bauters *et al.*, 2001). In addition to these common Gram-positive cocci and fungi, Buijssen *et al.* (2012) reported that lactobacilli were also predominant in the biofilms after examination of biofilms from 66 TESVs.

1.10   Culturomics: Diagnostic approaches for identification and quantification of biofilm community

New techniques in diagnostic methods have been developed continuously and used with the aim of optimizing the identification of pathogens in clinical microbiology. It is imperative that the techniques have been developed for rapid and easy identification can also be exploited to work towards methods for comprehensively isolating and identifying species from complex microbial communities. This is important as early detection and identification of microorganisms will greatly assist in providing the most appropriate antimicrobial therapy for better clinical outcome (Moussaoui *et al.*, 2010; Vlek *et al.*, 2012). Studies have shown that with the correct identification and the usage of appropriate antibiotic therapy will significantly reduce costs in the health care system (Garnacho-Montero *et al.*, 2003).

Most complex microbial ecosystems such as oral biofilm communities are so diverse that high-throughput microbial sequencing studies are needed to gain a detailed understanding of the microbial repertoire (Lagier *et al.*, 2012a). In order to provide more accurate identification of microbial species, the 'microbial culturomics' concept has been developed. It employs the use of multiple culture conditions with culture dependent identification by mass spectroscopy followed by the next gen sequencing of the new species cultured that allow high-throughput identification of rare and new species (Lagier *et al.*, 2012b).

1.10.1 Matrix-assisted laser desorption ionization time-of-flight mass-spectroscopy

Matrix-assisted laser desorption ionization time-of-flight mass-spectroscopy (MALDI-TOF MS) detects a large spectrum of highly abundant proteins universal to all microbes. The technique is based on the production of individual characteristic patterns and is capable of classifying organisms to the species level. This technique is currently the most suitable routine technique for bacterial and yeasts identification in the clinical microbiology diagnostic laboratory because it is accurate, rapid and inexpensive (Dhiman *et al.*, 2011; Lacroix *et al.*; Anderson *et al.*, 2014; Navrátilová *et al.*, 2016). This method can accurately identify fungi such as *C. albicans*, *C. glabrata*, *C. tropicalis* and many more (Dhiman *et al.*, 2011). When compared to conventional differentiation protocols that rely on lengthy incubation procedures and biochemical identification systems, identification of bacteria by MALDI-protocol is 1.45 days faster on average (Tan *et al.*, 2012). In a retrospective study by Eigner *et al.* (2009), a 95.2% consistency in the identification of bacterial species was shown between MALDI-Biotyper 2.0 and conventional biochemical identification. This finding was subsequently supported in 2013 when a study by Lacroix *et al.* (2013) reported that of 433 isolates requiring identification only 1.1% were misidentified by MALDI-TOF MS (Biotyper™ systems) compared to 10.2% using conventional methods.

The process of microbial identification by the MALDI-TOF MS BiotyperTM system is shown in Figure 1-6. Only a small amount of microbial biomass (10^4 to 10^6 CFU) is required for MALDI-TOF analysis. MALDI-TOF MS can identify bacterial species not just from a single colony of typical primary culture plates but also from positive blood

culture vials (Vlek *et al.*, 2012; Navrátilová *et al.*, 2016) and urine (Ferreira *et al.*, 2010).

MALDI-TOF is now extensively employed in the clinical setting, however, there are a number of limitations of the technique when differentiating between genetically similar organisms. Examples of this phenomenon include the incorrect identification of members of the mitis streptococcal group such as *S. pneumoniae* (Fan *et al.*, 2017), some *Shigella* species from *E. coli* and *C. albicans* from *C. dubliniensis* (Lacroix *et al.*, 2013).

1.10.2 Next-generation sequencing (Illumina MiSeq)

Advances in 'next generation' DNA sequencing technologies over the last two decades or so (Goodwin *et al.*, 2016) have enabled the production of large amounts of DNA sequence data rapidly at substantially lower costs compared to traditional Sanger sequencing (Mardis, 2013). The boom in the generation of genomic data, gene interactions and expression studies have changed the face of sequencing strategies with increased investment in high-throughput sequencing projects. Next generation sequencing (NGS) studies on microbial diversity have been performed on various different communities such as the oral microbiota (Diaz *et al.*, 2012a) and gut microbiome (Lagier *et al.*, 2015). Although, there are many challenges of NGS data analysis, techniques have been developed to reveal hidden patterns in sequencing, analysis and annotation to overcome these issues (reviewed by Tripathi *et al.* (2016)).

The Illumina MiSeq is commonly used for 16S rRNA gene sequencing as the technique requires shorter sequencing times than pyrosequencing and gives increased sequence read depth. The technique also generates the largest amount of sequencing data, up to 500 nucleotide reads of sequence, that are accessible from the MiSeq platforms at a relatively low cost (Caporaso *et al.*, 2012). As the TESV biofilm is a complex community, it is important to employ modern techniques for analysis such as NGS using the Miseq Illumina sequencing so that a more global representation of the species present can be obtained.

Pure subcultured colonies are added on a clean target plate

Samples are air dried after mixed with matrix solution
(cinnamic acid or a benzoic acid derivate)

Measurement in the MALDI-TOF MS is conducted

Analyte-specific mass spectrum is gained

Identification of species level through automatic matching of
the generated mass spectrum with spectra in the database

Figure 1-6: **The simplified workflow of MALDI-TOF** MS
(modified from Wieser and Schubert (2011))

1.11 Aims and objectives

From this introductory chapter, we can summarize that polymicrobial biofilms are the cause of many medical device-related infections. The ability to form a biofilm has been reported as an undesirable characteristic of microbial species and biofilm growth increases resistance towards anti-microbial drugs. The interactions between members of microbial communities can be synergistic and/or antagonistic, with coaggregation promoting biofilm formation. Microbial species, characteristic to different body locations, may interact and form strong and robust biofilm communities and cause diseases. Biofilms on TESVs are a particular problem for patients that require these valves for speech. Thus, the aim for this study was to understand the interactions of microbial species found together on TESVs by investigating the key mechanisms by which bacteria and *Candida* spp. interact. This included studies on the nature of the biofilm matrix in mixed-species biofilms.

The objectives for this study were: 1) To isolate different microbial strains from TESV biofilms and identify them using MALDI-TOF MS, and compare the data with culture independent analysis; 2) To investigate bacteria-candida interactions that promote biofilm information on TESVs; and 3) To reconstitute biofilms representing the natural microbiota of individual TESV biofilms in order to explore the interaction mechanisms further.

Chapter 2. Materials and Methodology

2.1 List of equipment

Application	Device	Manufacturer
Autoclave	Benchtop autoclave	Astell, Kent, UK
Incubation	Waterbath	Grant instruments, Cambridge, UK
Heat shock	Heating block	Flowgen bioscience Ltd, Nottingham, UK
Sonicator	B-12 Engisonic	Engis Ltd., Kent, UK
Gradient PCR	Thermocycler T100	Bio-Rad Laboratories Ltd., Hertfordshire, UK
qPCR	MJ Research DNA Engine Opticon 2	Bio-Rad Laboratories Ltd., Hertfordshire, UK
Centrifugation	J2-21	Beckman Coulter Ltd., High Wycombe, UK
	3K10	SciQuip Ltd., Shropshire, UK
Bench-top Centrifugation	MiniSpin®	Eppendorf UK Ltd., Stevenage, UK
Static Incubator	30 °C /37 °C	LTE Scientific Ltd., Oldham, UK
Orbital shaker	IKA KS 4000i control	IKA® England LTD., Oxford, UK
Anaerobic Incubator	Bug Box Plus	Ruskinn Technology Ltd., Bridgend, UK
Microplate reader	Synergy™ HT Microplate Reader	BioTek UK, Swindon, UK
Bead beater	Tissuelyzer LT	Qiagen, Manchester, UK
Nucleic acid concentration	NanoDrop® ND-1000	Thermo Fisher Scientific Ltd, Loughborough, UK
Determination of optical density	Libra S11	Biochrom Ltd., Cambridge, UK
Florescence microscopy	Zeiss Axioscope	Carl Zeiss Ltd., Cambridge, UK
Light/ Phase contrast microscopy	Leica DM 750	Leica Biosystems Newcastle Ltd. Newcastle upon Tyne, UK

2.2 Isolation and routine culture of microorganisms

2.2.1 Growth media and chemicals

For solidified medium or agar, 1.5 g bacto-agar (Melford Laboratories Ltd, Suffolk, UK) was added per litre before autoclaving. All media were autoclaved at 121 °C for 20 min and media containing agar were cooled to 45-50 °C in a water bath before pouring into petri dishes. All chemicals were obtained from Sigma Aldrich, Dorset, UK unless stated otherwise.

Brain heart infusion with yeast extract (BHYE) is a non-selective, enriched basic growth medium. BHYE included (per litre): 37 g brain heart infusion (Melford, UK) and 5 g yeast extract (Melford, UK) dissolved in distilled water (dH$_2$O).

Blood agar was prepared by adding fresh 15 mL sterile defibrinated blood or oxalated horse blood (TCS Biosciences, Buckingham, UK) per litre to cooled BHYE agar.

Chocolate agar was prepared using the same method as blood agar except that the fresh blood was replaced by lysed blood (prepared by slow heating defibrinated or oxalated horse blood to 75-80 °C until a reddish-brown colour).

Fastidious anaerobe broth (FAB) (Lab-M, Lanchashire, UK) was suspended in dH$_2$O (46 gL^{-1}) before autoclaving. Bacto-agar (15 gL^{-1}) was added to the broth before autoclaving to produce fastidious anaerobe agar (FAA). Once cooled, fresh 15 mL sterile defibrinated blood or oxalated horse blood was added.

de Man, Rogosa, Sharpe (MRS) medium provides a rich nutrient base that contains polysorbate (Tween 80), acetate, magnesium and manganese which are known to act as special growth factors for lactobacilli and lactic acid bacteria. This was prepared by adding 62 g MRS (Melford, UK) to dH$_2$O per litre.

Luria-Bertani (LB) broth is also a common growth medium used to culture bacteria. For *Escherichia coli* (*E. coli*) transformant isolation, LB solidified medium was enriched with 0.1 mgmL^{-1} ampicillin and 40 μgmL^{-1} X-gal.

Three types of media were used for selection and culture of fungi. Sabouraud dextrose (SD) (Melford, UK) (65 g) powder was suspended in dH$_2$O per litre. Yeast

extract peptone dextrose (YPD) broth was prepared by adding 20 g bacto-peptone, 10 g of yeast extract and 20 g dextrose per litre (Sherman, 2002) and a ready-made ChromID™ *Candida* agar (bioMérieux UK Ltd, Basingstoke, UK) is a chromogenic medium for selective isolation of yeasts and was used for distinguishing *C. albicans* and *C. tropicalis* during isolation. (Fricker-Hidalgo *et al.*, 2001).

Artificial saliva was prepared according to Pratten *et al.* (1998). This medium consisted of Lab-lemco (Melford, UK) 1 gL^{-1}, yeast extract (Melford, UK) 2 gL^{-1}, proteose peptone 5 gL^{-1}, type III hog gastric mucin 2 gL^{-1}, NaCl 0.35 gL^{-1}, KCl 0.2 gL^{-1} and $CaCl_2$ 0.2 gL^{-1}. After autoclaving at 121 ^oC for 20 min, filter sterilized 4% (w/v) urea was added to a final concentration of 500 mgL^{-1} and stored at 4 °C prior to use.

2.2.2 Sample collection

Tracheoesophageal speech valves (TESVs) that had ceased to function were collected from the Speech clinic, Freeman Hospital, Newcastle upon Tyne and transported to the Oral Microbiology laboratory, Newcastle University in reduced transport fluid (RTF).

RTF was adapted from Syed and Loesche (1972) and contained per litre: mineral salt (0.6 g K_2HPO_4, 1.2 g NaCl, 1.2 g $(NH_4)_2SO_4$, 0.6 g KH_2PO_4 and 0.25 g $MgSO_4$), 0.1M EDTA, 8 g Na_2CO_3 and 1 g dithiothreitol.

Each valve was treated with gentle ultrasonic treatment using a sonicating waterbath at 50 kHz for 60 s (Engisonic sonicating water bath, Engis Ltd., Kent, UK) and vortexed for 1 min in 1 ml of fresh RTF three times in separate tubes (modified technique from (Neu *et al.*, 1994a)). The samples were pooled before inoculation onto various growth media (refer to Table 2-1) (Holmes *et al.*, 2012).

An anaerobic environment was achieved by incubating cultures in an anaerobic incubator (Bug Box Plus, Ruskinn Technology Ltd., Bridgend, UK) with a gas mix consisting of 10% CO_2, 10% H_2 and 80% N_2 while aerobic samples were incubated in a static incubator (LTE Scientific Ltd., Oldham, UK).

Type of growth medium	Condition
Blood, chocolate and SD agar	37 °C, aerobically
FAA, chocolate, MRS agar	37 °C, anaerobically

Table 2-1: Growth media and conditions used during screening of TESV samples.

2.2.3 Glycerol stock

Through various screenings and observations, the colonies were picked, and axenic culture was established. In order to achieve this, the colonies were sub-cultured three times on agar. Colonies were transferred to 10 mL broth and cultured overnight. Any contamination was checked and resulting cultures were centrifuged (3 600 g) for 10 min at 4°C. One mL of growth medium mixed with 50% (v/v) glycerol (1:1 ratio) was added to pelleted samples before storage at -80 °C.

2.2.4 Total viable cell count

Serial ten-fold dilutions of cell suspensions were prepared in PBS for total viable cell counts. Twenty microliter spots of each dilution (10^3 to 10^6) were transferred to appropriate agar in triplicate. The plates were incubated aerobically or anaerobically at 37 °C for 24-48 h and a photo of each plate was taken using Canon IXUS 22HS camera. Colonies were counted using ImageJ (1.48v), an open source image processing program designed for scientific multidimensional images (http://imagej.nih.gov/ij/). The original number of colony forming units (CFU) ml^{-1} was calculated.

2.3 Identification of microorganisms

2.3.1 Gram staining and cell morphology

Colonies or liquid cultures were routinely checked by Gram staining. Dried, fixed smears of bacterial cells were initially stained with 2 % crystal violet. The unbound dye was washed away with tap-water before cells were counterstained with 1% Lugol's iodine (VWR International Ltd, Leicestershire, UK). Smears were decolorized for 30 s with 100% ethanol before 0.2% safranin O was flooded onto the slide. Cells were observed under a light microscope (Leica DM 750). The bacterial cells that retained the crystal violet stain are referred to as Gram positive, whereas the cells that were decolourized and appeared pink due to safranin counterstaining are designated Gram negative. In addition, microorganisms were categorized

accordingly to their microscopic morphology and their colonial appearance after growth on agar.

2.3.2 Identification using MALDI-TOF MS

Batches of screened TESV isolated samples were grown on a range of solidified growth media at appropriate growth conditions before sending for identification by Dr. Michael Ford, Freeman Hospital, Newcastle upon Tyne, UK using Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, Bruker Daltonik MALDI Biotyper (Bruker UK Ltd., Conventry, UK). For the MALDI-TOF mass spectrometry, samples were smeared onto a steel plate and matrix solution was added and allowed to dry. Species-level identification was generated when the smears were introduced to the fully automated mass spectrometer. A molecular fingerprint of microbes was generated when spectrum or profile of highly abundant proteins that are present in microbial species were detected and matched with an extensive library of spectra from known species. Score values were given in ranges (Table 2-2) to the identified species and samples were sorted.

Range	Description
2.300 ... 3.000	highly probable species identification
2.000 ... 2.299	secure genus identification, probable species identification
1.700 ... 1.999	probable genus identification
0.000 ... 1.699	not reliable identification

Table 2-2: **Score values for MALDI-TOF**.
Only samples with the range between 2 to 3 were considered to be reliably identified and were retained for glycerol stocks and storage at 80 °C.

2.3.3 Next generation sequencing

The microbial population in samples was assessed by sequencing of PCR-amplified 16S or 18S fragments according to Rostami *et al.* (2017) using Illumina® 16S/18S MiSeq™ and downstream bioinformatics by Dr. Scot E. Dowd (www.mrdnalab.com, Shallowater, TX, USA).

2.4   Phenotypic characteristics of TESV isolates

2.4.1   Production of H_2O_2 by isolated microorganisms

Hydrogen peroxide production in bacterial colonies was detected using the method of Jakubovics *et al.* (2008b). Briefly, 10 mL of overnight culture was spotted onto solidified THB medium and incubated for 24 to 48 h at 37 °C. Production of H_2O_2 by bacteria was tested by flooding the plate with 3 mL of detection reagent consisting of 100 mM potassium phosphate, pH 6.0, 20 U mL^{-1} horseradish peroxidase (HRP) and 1 mM 2, 2'-azino-bis (3-ethylbenzthiazoline- 6-sulphonic acid) (ABTS). Excess detection reagent was immediately removed, and plates were incubated at 25 °C for 15 min before imaging. In the presence of H_2O_2 and HRP, a purplish colour can be observed due to the conversion of ABTS to an ABTS radical cation. Accordingly, the production of H_2O_2 by microbial species was indicated by purple coloured colonies/growth spots.

2.4.2   Catalase test

For bacterial identification, a simple catalase test was used to differentiate between Gram-positive cocci as practised by NHS clinical microbiology laboratories according to UK Standards for Microbiology Investigations (Health-Protection-Agency, 2012). *Staphylococcus* are catalase-positive, while *Streptococcus* and *Enterococcus* are catalase-negative.

A few drops of 3% (v/v) H_2O_2 was added and mixed with a loop of a bacterial colony on a dry glass slide. A positive result was indicated by bubbling (within 5-10 s) that represented rapid production of oxygen and was seen by the naked eye.

2.4.3   Extracellular DNase activity using DNase test agar

The DNase activity of bacteria was assessed using DNase test agar (Melford, UK). All overnight cultures were grown in BHY or MRS broth, as appropriate. Five microliters of the overnight cultures were spotted onto the DNase test agar and incubated for 24 h at 37 °C in either an anaerobic chamber or aerobically. The plates were then flooded with 0.1% (w/v) toluidine blue and left for colour development for 2 min. Hydrolysis of DNA was distinguished by the development of pink zones around

the colonies in the presence of oligonucleotides, which are released when high molecular weight DNA is degraded (Schreier, 1969).

2.4.4 Germ tube formation of Candida spp.

One millilitre of *Candida* spp., grown overnight at 30 ^{o}C in YPD, was added to a fresh 8 mL of YPD and 1 mL of 10% (v/v) fetal calf serum to induce germ tube formation. The mixture was incubated in a rotating incubator (180 rpm) at 37 ^{o}C with sampling performed hourly for 6 h.

Samples (900 µL) were added to 100 µL 30% (w/v) paraformaldehyde, and fixed by incubation at room temperature for 20 mins. Cells were collected by centrifugation and washed three times with PEM (1 M Pipes pH 7.6, 10 mM $MgSO_4$ and 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)) . Cell suspensions were spread thinly to poly-L-lysine coated slides and allowed to air dry. Cells were fixed onto the slides by incubating in pre-chilled (to -20 ^{o}C) methanol for 6 min and chilled (-20 ^{o}C) acetone for 30 s before air drying. The slides were mounted using Vectashield$^®$ mounting medium containing DAPI (Vector Laboratories LTD., UK). Differential interference contrast (DIC) images were captured using a Zeiss Axioscope, with a 63x oil immersion objective, and Axiovision imaging system.

2.4.5 Coaggregation between isolated microorganisms

2.4.5.1   Visual coaggregation assay

The visual coaggregation method adapted from Jakubovics *et al.* (2008a) was used. Cells were cultured in growth media overnight, harvested and cultures were adjusted to OD_{600} = 1.0 in coaggregation buffer (1 mM Tris (pH 8.0), 150 mM NaCl, 0.1 mM $CaCl_2.2H_2O$, 0.1 mM $MgCl_2.6H_2O$ and 0.02% (w/v) NaN_3) (Metzger *et al.*, 2001).

Two samples in coaggregation buffer were mixed in equal ratio and vortexed for 10 s. The visual scoring was performed using a scoring system developed by Cisar *et al.* (1979) which rated coaggregation from 0 representing no coaggregation to 4+ the maximal coaggregation (clear supernatant) as shown in Table 2-3.

Score	Observation
4+	Formation of fast settling aggregates with the supernatant remaining water-clear
3+	Formation of fast settling aggregates, but with slightly turbidity supernatant
2+	The present of definite aggregates which did not settle immediately
1+	Finely dispersed aggregates in turbid background
0	No visible aggregates with no reduction in turbidity

Table 2-3: **Degree of coaggregation scoring** (Cisar *et al.*, 1979)

2.4.5.2 *Effect of different treatments on coaggregation*

In order to investigate coaggregation interactions further, cell suspensions at an O.D. of 1.0 at 600 nm were subjected to protease treatment by incubation with 0.45 mgmL^{-1} proteinase K suspended in PBS (pH 7.4) for 1 h at 37 ^{o}C. Samples were also heat treated at 85 ^{o}C for 30 min to denature proteinaceous adhesins.

Coaggregation assays were performed by suspending cells in coaggregation buffer containing either 80 mM L- lactose or L-arginine, as described by Min and Rickard (2009) to further determine if coaggregation could be blocked by simple sugars or amino acids.

2.4.5.3 *Microscopic analysis of coaggregation using fluorescent staining*

For microscopic visualisation of coaggregation, microbial cells suspended in 1 mL of coaggregation buffer were mixed with different dyes to stain each species individually prior to mixing them together. The dyes used were 1 mgmL^{-1} propidium iodide (PI), 50 µgmL^{-1} Concanavalin A Alexa Fluor® 488 (ConA) (Life Technologies Ltd, UK) or 2 µgmL^{-1} 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies Ltd, UK).

Each sample was incubated in darkness at room temperature for 5 min, washed with PBS, and harvested by centrifugation at 10 000 *g* for 1 min to rinse off excess dyes. The samples were then resuspended in 1 mL coaggregation buffer, mixed and vortexed for 10 s. The coaggregation between species was examined using a Zeiss Axioscope for visualisation of ConA (excitation 485 nm, emission 519 nm), PI (excitation 536 nm, emission 617 nm) and DAPI (excitation 358 nm, emission 461 nm).

2.4.6 Impact of stress on Candida spp.

2.4.6.1   Screening of environmental stressors using the spot test

Isolated *Candida* spp. were screened for resistance to different environmental stresses, including oxidative (hydrogen peroxide (H_2O_2), menadione and tert-Butyl (t-BOOH) hydroperoxide), osmotic (sorbitol and sodium chloride) and heavy metal ($CdSO_4$ and NaAs) stresses.

Candida spp. were cultured for 24 h at 30 °C in YPD broth. Cells were diluted to an OD_{660} of 0.2 in fresh YPD and grown until exponential phase was reached ($OD_{660} = 0.6$). Cells were then diluted to an OD_{660} of 0.2 in fresh YPD. Ten-fold serial dilutions from each sample were spotted onto YPD plates containing the indicated stress-inducing compounds. The plates were incubated at 30 °C for 24 h. The growth of the colonies on the agar was compared.

The stress inducing agents were prepared using stock solutions of 30% (v/v) H_2O_2, 100 mM tBOOH, 100 µM menadione or 100 µM diamide. Stock solutions of 4M sorbitol and 4 M NaCl were used to induce osmotic stress and cation stress respectively, and heavy metals (0.5 M $CdSO_4$ and 0.5 M NaAs) were employed as heavy metal stock solutions. The final concentration of such agents is indicated in the text.

2.4.6.2   Quantitative analysis of Candida cell survival on H_2O_2 exposure

To measure the ability of *Candida* cells to survive short-term acute H_2O_2 stress exposure, a liquid assay of survival is more quantitative than the spot tests. *Candida* spp. were cultured for 24 h at 30 °C in YPD broth. Cells were diluted to an OD_{660} of 0.2 in fresh YPD and grown until exponential phase ($OD_{660} = 0.6$). Such cells were incubated with a final concentration of 10 mM H_2O_2 in a 30 °C rotary incubator (180 rpm). Samples were removed at 0, 30, 60, 90 and 120 min, diluted (1:3300) and spread onto YPD agar. The plates were then incubated overnight at 30 °C. Colonies were enumerated the next day and survival curves plotted.

2.5 Molecular biology methods

2.5.1 DNA extraction from microbial cells

DNA extractions were performed using two different kits; the ZR fungal/bacteria DNA miniprep (Cat. No. D6005) (Cambridge Bioscience, Cambridge, UK) and MasterPure™ DNA purification kit (Cat. No. MCD85201) (Cambio Ltd, Cambridge, UK) according to the manufacturer's instructions.

In order to improve purity and yield, a modified method derived from both two kits was developed. Ten mL of overnight microbial cultures in appropriate growth medium were centrifuged at 13 000 g for 10 min. The resultant pellets were mixed with 150 µL of pre-warmed spheroplasting buffer (20 mM Tris-HCl, pH 6.8; 10 mM MgCl$_2$, 26% w/v raffinose.5H$_2$O) containing 250 µgmL^{-1} lysozyme and 50 U mutanolysin (reconstituted to 10,000 U/mL) and incubated for 30 min at 37 °C. Cells were lysed by transferring the sample mixture into a ZR BashingBead™ lysis tube (ZR fungal/bacteria DNA miniprep), adding 150 µL of T&C 2x lysis solution (MasterPure™ DNA Purification Kit) and 1 µL of Proteinase K, and bead beating in a Tissuelyzer LT (Qiagen, Manchester, UK) for 5 min at 50 Hz. Samples were incubated for 30 min at 65 °C to optimise the Proteinase K reaction. RNase A (5 µgmL^{-1}) (MasterPure™ DNA Purification Kit) was added to the samples and incubated for 30 min at 37 °C. Samples were centrifuged at 10 000g for 1 min to obtain the cell lysates (in supernatant).

DNA extraction was continued using the ZR fungal/bacteria DNA miniprep according to manufacturer's instructions. To clarify crude cell lysates, 400 µL of the supernatant was transferred into a Zymo-spin IV spin filter and centrifuged at 7 000g for 1 min. Filtrate containing 1 200 µL of fungal/bacterial DNA binding buffer was added into a Zymo-spin IIC column for binding and centrifuged at 10 000 g for 1 min. For washing steps, 200 µL DNA pre-wash buffer was added and centrifuged followed by 500 µL Fungal/bacterial DNA wash buffer in the same column as the filtrates and centrifuged again. A DNA elution step was performed by adding 15 µL of DNA elution buffer into the column and incubated for 5 min at room temperature before centrifugation at 10 000 g for 30 s. Samples were then stored in 1.5 ml microcentrifuge tubes at -20 °C. The DNA concentration was determined using a Nanodrop® ND-1000 spectrophotometer (ThermoFisher scientific Ltd, Loughborough, UK).

2.5.2 Primer design

Primers (UnivF and UnivR) and probe (UnivP) targeting the 'universal' 16S rRNA gene for bacteria were originally designed by Nadkarni *et al.* (2002). For *C. albicans*, probe (Ca_P) and primer (Ca_F1) were originally developed by Guiver *et al.* (2001), with a single nucleotide modification in the reverse primer (Ca_R1), as reported by Yassin *et al.* (2016). These primers match *C. albicans* VPSA1 internal transcribed spacer 2 (ITS2), (NCBI Genbank Reference Sequence: KJ739863.1). For *C. glabrata* primers (CglbF and CglbR) and probe (CglbP) were designed by Guiver *et al.* (2001) to target *C. glabrata* CBS 138 internal transcribed spacer 1 (ITS1) in Chromosome L (NCBI Genbank Reference Sequence: AY198398.1).

The various probes and primers used were checked for complementarity against the genome sequences that are available through NCBI Genbank by using SnapGene® software version 3.1.4 (GSL Biotech LLC). TaqMan® (sequence-specific DNA) probes consisting of oligonucleotides modified with a fluorescence reporter dye (5' end) and a fluorescence quencher (3' end) were used and details are given in Table 2-1. Oligonucleotide primers and probes were ordered and synthesised by Eurogentec (Eurogentec, Liège, Belgium).

2.5.3 Polymerase chain reaction and purification of PCR products

Overnight microbial cultures (1 µL) were mixed with 20 µL GeneReleaser® (Bioventure, Inc., USA) and the thermal cycler lysis protocol was performed at 65 °C for 30 s; 8 °C for 30 s; 65 °C for 90 s; 97 °C for 180 s; 8 °C, 60 s; 65 °C, 180 s; 97 °C, 60 s; 65 °C, 60 s and hold at 80 °C using a T100 Thermal Cycler (BioRad, Hertfordshire, UK) to release DNA from the sample. DNA fragments were amplified using different primers (refer to Table 2-4) according to species/samples. PCR reactions were performed using 0.25 µM forward primer, 0.25 µM reverse primer, 25 µL 2X ReddyMix PCR Master Mix with 1.5 mM $MgCl_2$ (ThermoFisher scientific Ltd, UK), 1 µL DNA template with total reaction volumes of 50 µL.

The PCR program started at 94 °C for 2 min and 35 cycles of denaturation, 94 °C for 10 s; annealing, 55-58 °C for 30 s; elongation 68 °C for 60 s and 68 °C for 7 min, finally holding at 4 °C. The PCR products were cleaned using the QIAquick PCR Purification kit (Qiagen, Manchester, UK) and eluted in 20 µL elution buffer. The

concentration of the PCR products was measured using the Nanodrop® ND-1000
with the DNA purity estimation based on absorbance readings at 260 nm and
280 nm, with a ratio of ~1.8-2.0 considered acceptable.

2.5.4 Agarose gel electrophoresis (AGE)

DNA samples from PCR, qPCR or restriction digest reactions were analysed by
electrophoresis through 1% agarose gels to estimate the size and purity of DNA
fragments. 1% agarose gel was prepared by boiling as required in Tris-acetate-
ethylenediaminetetraacetic acid (TAE) buffer (40 mM Tris/acetate, 1 mM EDTA, pH
8.0). Five µL of GelRed™ Nucleic Acid Gel Stain (10 000x in DMSO) (Cambridge
Bioscience Ltd, UK) was added to the gel in order to visualise DNA. DNA products
were mixed 1:5 with 5x DNA Loading Buffer (Bioline Reagents Ltd., London, UK) for
loading on the gel. HyperLadder 1 kb Plus (250-12,007 bp) (Bioline, UK) was used
as DNA molecular weight marker for product size reference. The gel was run at a
constant voltage of 80 V, for 90-120 min, using a Bio-Rad Power Pac 300 and then
transferred to a G:BOX Transilluminator (Syngene, Cambridge, UK) to capture
images at 5.5 Mpixel using GeneSnap software (Syngene, UK).

 DNA samples with custom primers were sent for sequencing to an external
company (Eurofin genomics, Ebersberg Germany) for identification of unknown
products. Sequences were aligned using SnapGene® software version 3.1.4.

	Label	Primers (5′-3′)	References
Universal 16s	63f	CAG GCC TAA CAC ATG CAA GTC	Marchesi *et al.* (1998)
	1387r	GGG CGG WG TGT ACA AGG C	
	UnivF	TCCTACGGGAGGCAGCAGT	Nadkarni *et al.* (2002)
	UnivR	GGACTACCAGGGTATCTAATCCTGTT	
***C. albicans* ITS2**	Ca_F1	GGGTTTGCTTGAAAGACGGTA	Guiver *et al.* (2001)
	Ca_R1	TGAAGATATACGTGGTAGACGTTA	Yassin *et al.* (2016)
***C. glabrata* ITS1**	CglbF	TTTCTCCTGCCTGCGCTTAA	Guiver *et al.* (2001)
	CglbR	ACGCACACTCCCAGGTCTTT	
TaqMan® probes (5′-3′)			
Universal 16s	UnivP	6-HEX-CGTATTACCGCGGCTGCTGGCACAC-TAMRA	Nadkarni *et al.* (2002)
***C. albicans* ITS2**	Ca_P	6-FAM-ACCTAAGCCATTGTCAAAGCGATCCCG-TAMRA	Guiver *et al.* (2001)
***C. glabrata* ITS1**	CglbP	YY-AGAACACCCACCAACCGCGCA-QXL570	Guiver *et al.* (2001)

Table 2-4: **Forward and reverse primers and probes used in this study**.
Note: W can be either weak A or T http://www.genome.jp/kegg/catalog/codes1.html

2.5.5 Generation of plasmid by TOPO cloning

2.5.5.1 Plasmid transformation

Plasmids containing qPCR target sequences for use as standards in qPCR reactions were created using Invitrogen TOPO cloning kit (Life Technologies Ltd, UK) for cloning PCR-amplified fragments into pCR2.1-TOPO vector.

Generation of plasmid was carried out by direct insertion of Taq polymerase-amplified PCR products (refer to Section 2.5.3) into a plasmid vector. The reaction contained 3 μL fresh PCR product, 1 μL salt solution (1.2 M NaCl, 0.06 M MgCl$_2$), 1 μL pCR2.1 vector, and distilled water to a total volume of 5 μL. Samples were incubated at room temperature (~23 °C) for 10 min.

Each product of the topoisomerase reactions (1–5 μL) was added directly into a 50 μL vial of One Shot® TOP10 chemically competent *E. coli* cells for transformation and mixed by tapping gently. The cells were incubated on ice for 30 min and heat shocked at 42 °C (heating block, Flowgen Biosciences Ltd., UK) for 30 s followed by 2 min incubation on ice. 250 μL of pre-warmed S.O.C medium (Life Technologies Ltd, UK) were added to each vial before incubation in a shaking incubator (225 rpm) at 37 °C for 1 h. 200 μl of cells were spread onto LB agar with ampicillin and X-gal. Plates were incubated overnight at 37 °C. White colonies represented transformed cells with vectors containing recombinant DNA and blue colonies were cells transformed with non-recombinant plasmids.

Once the correct plasmids were identified, large scale preparations were made using the Qiagen plasmid midi kit (Qiagen). For this, 10 μl of -80 °C cultures were transferred to universal bottles containing 5ml LB/Amp broth. The cultures were incubated at 37 °C with shaking at 200 rpm for 8 h. Cells were harvested by centrifugation at 5,000g for 10 min at 4°C, and plasmids were extracted according to the manufacturer's instructions. The purified plasmids were checked using the NanoDrop spectrophotometer and agarose gel electrophoresis and stored at -20°C.

2.5.5.2 Analysis of plasmid (restriction digestion)

Restriction enzyme digestions were performed on plasmid DNA extracts to check for the correct insertion of gene fragments of interest. Digestion reactions were carried out with the following reagents: 2 µL plasmid DNA product (up to 1 µg), 2 µL buffer, 1.6 µL restriction enzyme and distilled water to 20 µL of total reaction volume. The reactions were incubated for 1 h at 37 °C and inactivated at 80 °C on a heating block (Flowgen bioscience Ltd, UK) for 15 min. DNA fragments were analysed by AGE (refer to Section 2.5.4).

Restriction Enzyme	Recognition Site[a]	NEBuffer[b]
*Bgl*II	5' A▼GATCT 3' 3' TCTAG▲A 5'	NEBuffer 3.1 (100 %)
*Not*I	5' GC▼GGCCGC 3' 3' CGCCGG▲CG 5'	*NEBuffer 3.1 (100 %)*
*Dra*III-HF®	5' CACNNN▼GTG 3' 3' GTG▲NNNCAC 5'	*CutSmart® Buffer (100%)*
*Xba*I	5' T▼CTAGA 3' 3' AGATC▲T 5'	*CutSmart® Buffer (100%)*

Table 2-5: **Restriction enzymes used during this study**.
[a]Triangles indicate sites of digestion. [b]Buffer used, according to the New England BioLabs nomenclature. Percentage in brackets indicates the activity of each restriction enzyme in the buffer used.

2.5.6 DNA concentration with Quant-iT™ PicoGreen® dsDNA Assay

A standard curve of DNA concentration employed Quant-iT™ PicoGreen® dsDNA assay kit (Fisher Scientific UK Ltd, Loughborough, UK) in a black 96-well flat-bottomed plate. Lambda DNA (2 µgmL^{-1}) was diluted from a range of

1 000 ngmL^{-1} to 1 ngmL^{-1} in TE buffer to develop a standard curve. Two hundred-fold diluted PicoGreen® dye in TE buffer was added to the λ DNA standards and DNA samples of equal volume. The plate was incubated at room temperature for 5 min and protected from light.

The fluorescence was measured at excitation 480 ± 10 nm/ emission 520 ± 10 nm in a Synergy™ HT microplate reader (BioTek UK). Concentrations of DNA samples were calculated from the DNA standard curve using Microsoft® Excel 2013 (Microsoft® Office professional plus 2013, Microsoft®).

2.5.7 Real-Time quantitative polymerase chain reaction

Probe based real-time quantitative polymerase chain reaction (qPCR) was carried out on whole genome DNA products. 1.0 μL of DNA sample with qPCR reaction mix (1 μL 2.5 μM forward primer, 1 μL 2.5 μM reverse primer, 0.5 μL of 2.5 μM probe, 6.25 μL of 2X Premix Ex Taq™ (Takara Bio Europe, Saint-Germain-en-Laye, France), 2.75 μL of dH$_2$O) was pipetted into frosted, non-skirted, low profile 96-well qPCR plates (Eurogentec Ltd, UK), and sealed with an optical Microseal 'B' Adhesive seal (Bio-Rad Laboratories Ltd., Hertfordshire, UK).

Reactions were performed on a MJ Research DNA Engine Opticon 2 (Bio-Rad Laboratories Ltd., UK), using the following programme: initial denaturation at 95 °C for 2 min, denaturation at 95 °C for 5 s, annealing/extension at 60 °C for 30 s, plate read and the cycle was repeated 40 times (Yassin *et al.*, 2016). All the reactions were carried out in triplicate with ten-fold serially diluted reference standards and blank. DNA concentrations of plasmids were used as reference standards for absolute quantification (refer to Section 2.5.6). Standard curves and AGE were performed to validate the qPCR experiments. The final analysis was based on the mean of the three reactions. Amplification efficiencies (between 90-105%) were determined from the slope of the log-linear portion of the standard curve according to MIQE guidelines (Bustin *et al.*, 2009).

$$\text{qPCR efficiency} = 100*(10^{-1/\text{slope}} - 1)$$

Logarithm of the initial template concentration (the independent variable) is plotted on the x axis and Cq (the dependent variable) is plotted on the y axis

Bacteria	16s rRNA copy	Reference strains
Porphyromonas gingivalis	4	*Porphyromonas gingivalis* W83
Lactobacillus fermentum	5	*Lactobacillus fermentum* IFO 3956
Ochrobactrum anthropi	4	*Ochrobactrum anthropi* ATCC 49188
Staphylococcus aureus	4	*Staphylococcus aureus* 55/2053
Streptococcus oralis	4	*Streptococcus oralis* Uo5
Staphylococcus epidermidis	6	*Staphylococcus epidermidis* RP62A
https://rrndb.umms.med.umich.edu/, accessed on 18th April, 2016 (Stoddard *et al.*, 2015)		

	Regions		Copies	Reference strains	References
C. albicans	ITS2	diploid	110	SC5314	Jones *et al.* (2004)
C. glabrata	ITS1 (ChrL)	haploid	118	CBS138/ ATCC2001	Maleszka and Clark-Walker (1993)

Table 2-6: **Copy number of qPCR target regions for bacteria and *Candida* spp.** ITS2 (*C. albicans*) spacer region, ITS1 (*C. glabrata*) spacer region and 16S rRNA for all the bacteria were used as targets and corrections were made for gene copy number. Representative genome sequences of *C. albicans*, *C. glabrata*, and bacteria have been determined as shown in Table 2-3 . The quantity of template DNA in each sample was calculated in accordance with MIQE guidelines (Bustin *et al.*, 2009) using statistical software Microsoft® Excel 2013.

2.6 Biofilm modelling

2.6.1 Standardization of inoculae for biofilm models

Five microliters of frozen stock were inoculated into 10 mL media and grown overnight at 37 °C either aerobically or anaerobically. An estimation of sample cell number was obtained using viable cell count (refer to Section 2.2.4). Three *C. albicans* strains were isolated, one each from TESVs 8, 11 and 16. The *C. glabrata* and bacterial samples were obtained from TESV 16 isolates.

2.6.2 Static biofilm formation

A static, mixed species biofilm was used to study the interactions between *Candida* spp. and the bacteria isolated from speech valves. A 6-well plate containing 3 mL of artificial saliva was used to grow the biofilm at 37 °C for up to 48 h. A total of 3 mL containing 10^9 cells of *C. albicans* and *C. glabrata* and/or 10^8 cells of each bacteria isolated from TESV in artificial saliva were added into wells. Each biofilm experiment was performed in triplicate. The plate was covered and incubated at 37 °C in a humid environment.

A change of medium was performed after 24 h for 48 h biofilms. Used medium was discarded on the collection day, and wells were washed once with PBS. One mL of PBS was added before scraping the biofilm for collection. Samples were centrifuged at 13 000*g* for 10 min at 4 °C and the supernatant was discarded. The pellets were snap frozen in liquid nitrogen and stored at -80 °C.

2.6.3 Biofilm mass quantification

Biofilm mass quantification of biofilms was done using crystal violet assay and qPCR (described in Section 2.5.7). The crystal violet assay employed a colorimetric method for measuring the formation of biofilm in 96-well plates as described by O'Toole (2011). A total of 200 µL of 10^8 bacterial cells and 10^9 of *Candida* spp. in artificial saliva were added into wells of a 96-well plate. Each sampling was performed in triplicate. The plate was covered and incubated at 37 °C in a humid environment.

After 24 h, the medium was discarded and washed with PBS. 100 µL of 0.5% (w/v) crystal violet was added to each well and incubated for 15 min at room temperature.

The wells were rinsed three times with PBS to remove excess unbound crystal violet. 100 µL of 7% (v/v) acetic acid were added. The absorbance reading at A_{570}, representing the amount of biofilm growth of the community over time, was measured using a Synergy™ HT microplate reader.

2.7 Protein analysis

2.7.1 Protein extraction from biofilm

Samples were collected from 48 h *Candida* biofilms, either with or without bacteria. Cells were harvested by centrifugation at 3000 *rpm* for 5 min at 4 °C prior to snap freezing in liquid nitrogen.

Samples were thawed on ice, washed in 1 mL of ice-cold protein lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP_4O (v/v), and 10 mM imidazole) containing protease inhibitors (1 mM PMSF, 0.07 TIU/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin) and phosphatase inhibitors (2 mM Sodium vanadate (Na_3VO_4) and 50 mM NaF). Cells were collected by centrifugation, resuspended in 100 µl of lysis buffer and pipetted into a tube containing 750 µL of cold glass beads. Cells were disrupted using a bead beater at 50 kHz for 2 times of 30 s with 1 min on ice in between. Lysates were collected, and cell debris was removed by centrifugation at 13 000 rpm for 10 min at 4 °C.

2.7.2 Determination of protein concentration

Protein concentrations were determined using Bradford protein assay (Coomassie Bradford reagent) (ThermoScientific Ltd., UK) according to the manufacturer's instructions. Samples were prepared to the desired protein concentration, and 2x SDS loading dye (62.5 mM Tris pH 6.7, 2% SDS (w/v), 50% glycerol) with 50 µL ß-mercaptoethanol was added and stored at -20 °C.

2.7.3 SDS-PAGE and Western blotting

Protein extraction from mixed species biofilms was carried out (Section 2.7.1) and 25 µg of protein extracts were analysed by 8% SDS-PAGE and western blotting. Protein samples were analysed by electrophoresis through 8% or 10% SDS polyacrylamide gels (Laemmli, 1970). Proteins from polyacrylamide gels were

transferred to nitrocellulose membrane (Protran®, Schleicher & Schuell Bioscience, DE), and blocked with bovine serum albumin (BSA) (10% BSA (w/v) in TBST; 1x TBS (1 mM Tris HCl (pH 8), 15 mM NaCl) and 0.01% Tween 20. Membranes were probed by incubating with primary antibodies (details below), diluted 1:1 000 in 5% BSA in TBST, overnight at 4 °C. The membranes were washed 5 x 5 min with TBST prior to incubation at room temperature for 45 min with HRP-conjugated secondary antibodies (diluted 1:2 000 in 5% BSA in TBST). Blots were washed 3 x 5 mins with TBST prior to development. Film development of the membrane was done using the ECL™ Western blotting detection system (GE Healthcare Life Sciences, Buckinghamshire, UK) and Fuji Medical X-ray film.

Phosphorylated Hog1 was detected with an anti-phospho-p38 antibody (New England Biolabs, Massachusetts, USA) (Smith *et al.*, 2004) and HRP-conjugated anti-rabbit secondary antibody (Sigma aldrich, UK). Total Hog1 level in the samples were determined by stripping the blots and probing with an anti-Hog1 antibody (Santa Cruz Biotechnology, Texas, USA) and HRP-conjugated anti-rabbit secondary antibody (Sigma aldrich, UK). The size of *C. albicans* Hog1 is 43 kDa while *C. glabrata* Hog1 is 50 kDa.

2.8 Statistical analysis

All samples were tested for normality using Shapiro-Wilk test to determine the distribution of the data. For normally distributed data, Student's t-test, paired t-test and Bonferroni test for a repeated measures ANOVA was used when appropriate and for non-normal distributions, a non-parametric test (Kruskal-Wallis test) was employed.

All data were calculated as the mean ± standard deviation (biological triplicate) of at least three experiments (experimental triplicate). Differences were considered statistically significant at $P < 0.05$ when comparing between groups. Data analyses were done using statistical software IBM SPSS Statistics ver. 23 and all graphs were generated using Sigma-plot® ver.12.5.

Chapter 3. Biofilm Composition of Tracheaesophageal Speech Valves

3.1 Introduction

Tracheaesophageal speech valves (TESVs) are currently widely used for rehabilitation in tracheostomy patients in order to restore speech function. However, there is a need to replace the devices frequently as the average lifespan of TESVs is only between 108 to 207 days (Hancock *et al.*, 2013; Kress *et al.*, 2014). Patients' activities are interrupted when there is leakage or valve dysfunction because they have difficulty in breathing and speaking. As well as causing inconvenience and discomfort to the patients, TESV replacement is expensive. It has been estimated in the Newcastle area, the annual cost per patient is £530-£670 (Owen and Paleri, 2013). Valve failure may be caused by microbial colonization leading to inadequate valve closure, or non-microbial physical defects, and requires immediate replacement (Rodrigues *et al.*, 2007). A 5-year retrospective study in the Netherlands showed that obstruction of TESVs leading to leakage through the valve is the main reason for replacement of the device as shown in Figure 3-1 (Oosterhof *et al.*, 2005).
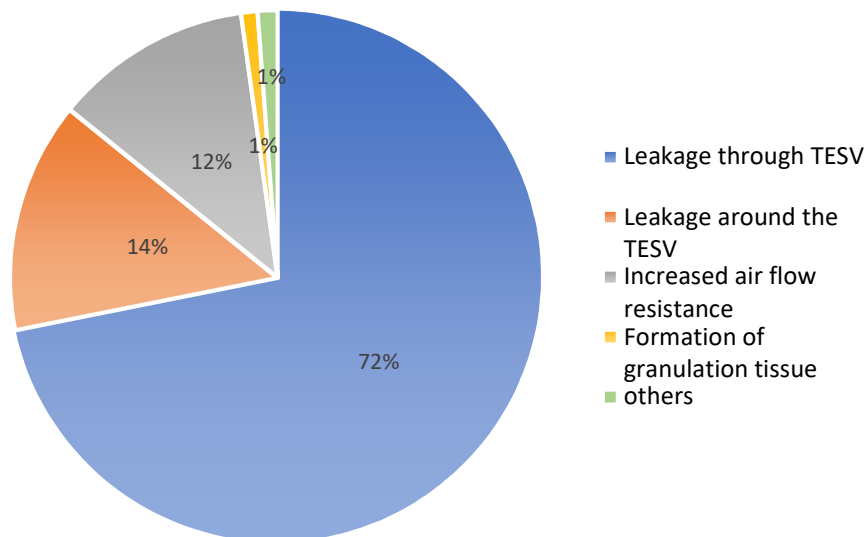
Figure 3-1: **5-year retrospective study conducted in Groningen with 138 patients (746 valve replacements)** (Oosterhof *et al.*, 2005).

Most members of the microbial communities in biofilms on failed TESVs, such as *Candida* spp. and *Streptococcus* spp., are considered to be opportunistic pathogens (Neu *et al.*, 1994b; Bauters *et al.*, 2001; Honraet *et al.*, 2005). The location of the valve makes it particularly susceptible to be colonized by oral pathogens (Bertl *et al.*, 2012) and studies showed that *Candida* spp. are capable of attaching to TESV materials (Rodrigues *et al.*, 2007; Estivill *et al.*, 2011; Buijssen *et al.*, 2012; Holmes *et al.*, 2014). It has been reported that up to 83% of extracted valves presented with polymicrobial fungal/ bacterial colonization (Tićac *et al.*, 2010; Somogyi-Ganss *et al.*, 2017). Amongst fungi, *C. albicans* is considered as the most prevalent species to colonize medical devices implanted in patients including TESVs (Tićac *et al.*, 2010; Buijssen *et al.*, 2012; Shakir *et al.*, 2012). However, a recent study showed that *C. tropicalis* and *C. glabrata* can be co-isolated the biofilm together with *C. albicans* (Somogyi-Ganss *et al.*, 2017).

It has been known for almost 40 years that *Candida* spp. are able to colonize silastic materials such as those used in the production of speech valves (Mahieu *et al.*, 1986; Ell, 1996; Brand, 2012). These fungi can also bind to host cell receptors (Holmes *et al.*, 2014) and penetrate tissues (Cavalcanti *et al.*, 2015) as well as implanted device surfaces (Estivill *et al.*, 2011). Ell (1996) observed finger-light projections at the edge of a dysfunctional silastic valve. He described what he saw under the light microscope after staining with toluidine blue as 'erosion of a malignant ulcer or cancerous growth into its basal stroma and give an impression of aggressive nature of the fungal invasion of silastic'.

Biofilm formation by *Candida* spp. in a model system is initiated by the attachment of yeast cells to the device surface. Colonization continues by the proliferation of yeast cells and the establishment of pseudohyphae and hyphae in the presence of an extracellular matrix (Hawser *et al.*, 1998; Ramage *et al.*, 2002a; Douglas, 2003; Cavalcanti *et al.*, 2015). Bacterial species such as *S. oralis* and *S. gordonii* have been observed attaching to hyphae of *C. albicans* (Diaz *et al.*, 2012b; Nobbs and Jenkinson, 2015). The presence of bacteria, along with alterations in host immunity or changes in the environment, can influence the course of *C. albicans* infection (Pappas *et al.*, 2004). Nobbs and Jenkinson (2015) showed that in polymicrobial interactions, *C. albicans* exhibited higher proportions of hyphal forms relative to yeast cells and suggested that the presence of bacteria promoted biofilm formation

and increased its virulence which is supported by a number of other studies (Nobile and Mitchell, 2006; Fox *et al.*, 2014).

Interactions between different microbial species in biofilms contribute to the development of increased population complexity. Biofilm complexity can provide protection to the microbial community members from external stresses such as desiccation and antimicrobial substances. *Candida* cells in biofilms are less susceptible to antimicrobial agents (Kuhn and Ghannoum, 2004). In addition, *Candida* has been shown to provide protection to the bacteria by the secretion of cell wall polysaccharides that encase the community (Kong *et al.*, 2016). A study by Romano and Kolter (2005) demonstrated beneficial interactions in polymicrobial biofilms such as the ability of the fungus to metabolize available glucose that result in environmental pH changes that provide a favourable effect on bacterial physiology and survival. However, there is a lot of work to be done before we can fully understand the key intermicrobial interactions that occur in polymicrobial biofilms.

Most previous studies on the microbiological analysis of mixed-species TESV biofilms have employed culture dependent techniques to analysis the microbiota (Neu *et al.*, 1994a; Eerenstein *et al.*, 1999; Leunisse *et al.*, 2001; Millsap *et al.*, 2001; Bauters *et al.*, 2002; Elving *et al.*, 2002; Honraet *et al.*, 2005). These are inadequate for determining the full genetic diversity of complex microbial populations as it is well-known that many species are currently unculturable in the laboratory setting. Recent studies have implemented a molecular approach, based on polymerase chain reaction (PCR) using extracted DNA from samples, for species identification (Bertl *et al.*, 2012; Buijssen *et al.*, 2012; Somogyi-Ganss *et al.*, 2017). A review of the literature indicates that there is a dearth of studies using a culturomic approach; that consists of culturing, MALDI-TOF MS and next generation sequencing (NGS) for isolation and identification of TESV polymicrobial biofilm populations.

The aim of this chapter was to identify the microbial community members of selected TESVs using both culture independent techniques (Next generation sequencing) to analyse the microbiota, including unculturable species, and routine microbiology techniques (culture dependent method) and to obtain representative isolates that can form the basis for experiments to enable increased understanding of the community. The objectives were; 1) to sequence DNA extracted from TESV biofilm samples for microbial species identification using Illumina® 16S/18S MiSeq™, 2) to isolate and

identify microorganisms from a number of failed TESVs using routine microbiological techniques, and 3) to screen the isolated communities for key phenotypic properties relating to their ability to thrive in multispecies communities.

3.2   Collection and Examination of TESVs

Seventeen discarded TESVs that had become dysfunctional due to the accumulation of biofilm were collected from the ENT clinic at the Freeman Hospital, Newcastle upon Tyne (Figure 3-2). Of these, 6 TESVs were used to optimize the culturomics method and the remainder were used to generate sampling data. The workflow used for sample collection, processing, identification and storage is illustrated in Figure 3-3. An extracted TESV with macroscopically visible biofilm (brownish spots) on the surface was sent for imaging by FE-SEM performed by Leon Bowen, Durham University, Durham UK. A mixture of different morphotypes such as rods, cocci and hyphae of various species were observed (Figure 3-4). Tangled, mesh-like structures of extracellular material surrounding the microbial cells were also seen.
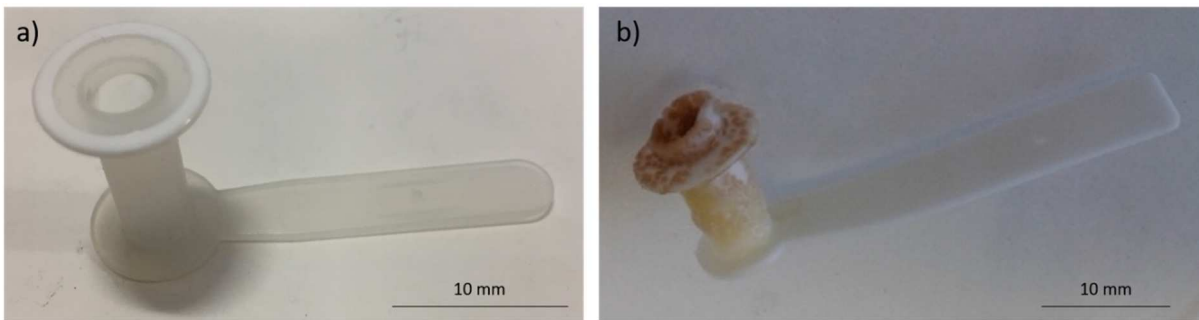
Figure 3-2: **Different types of indwelling TESV used in this study**.
a) Clean unused Blom-Singer® Classic indwelling TESV and b) Extracted Blom-Singer® low pressure indwelling TESV covered with biofilm (brownish spots)
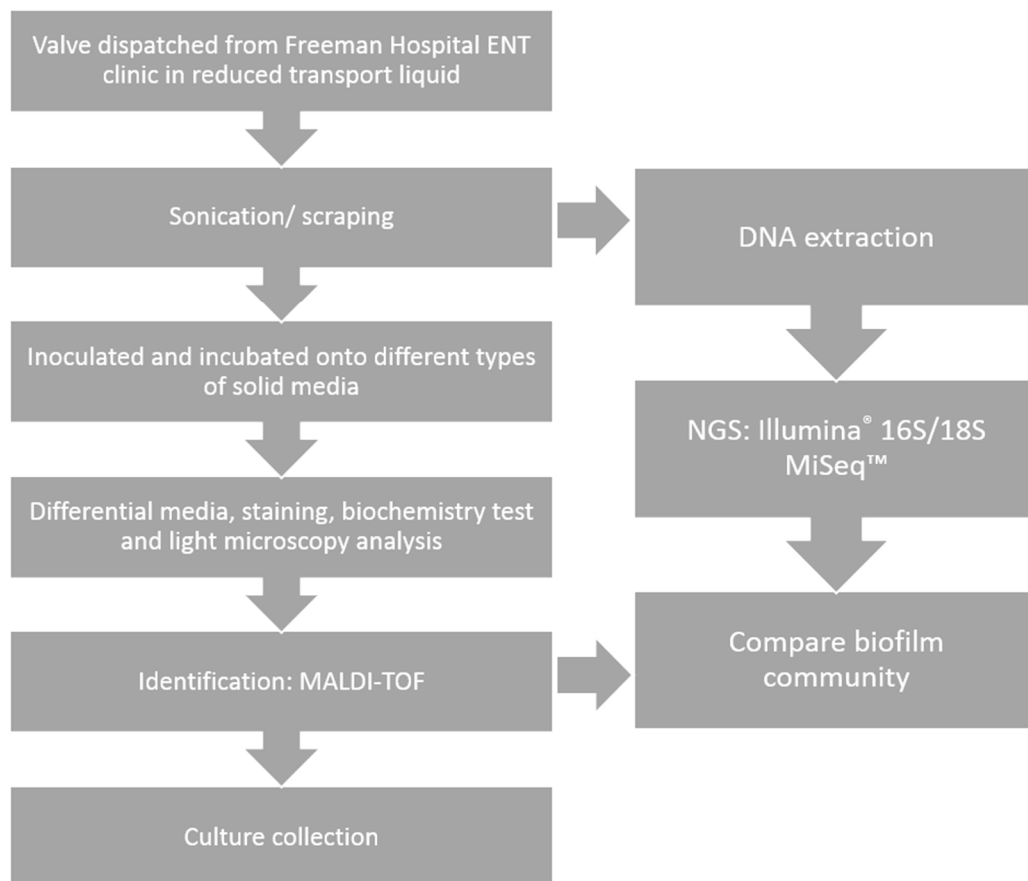
Figure 3-3: **The workflow for TESV biofilm collection and handling**.
Details of each step are provided in Chapter 2.
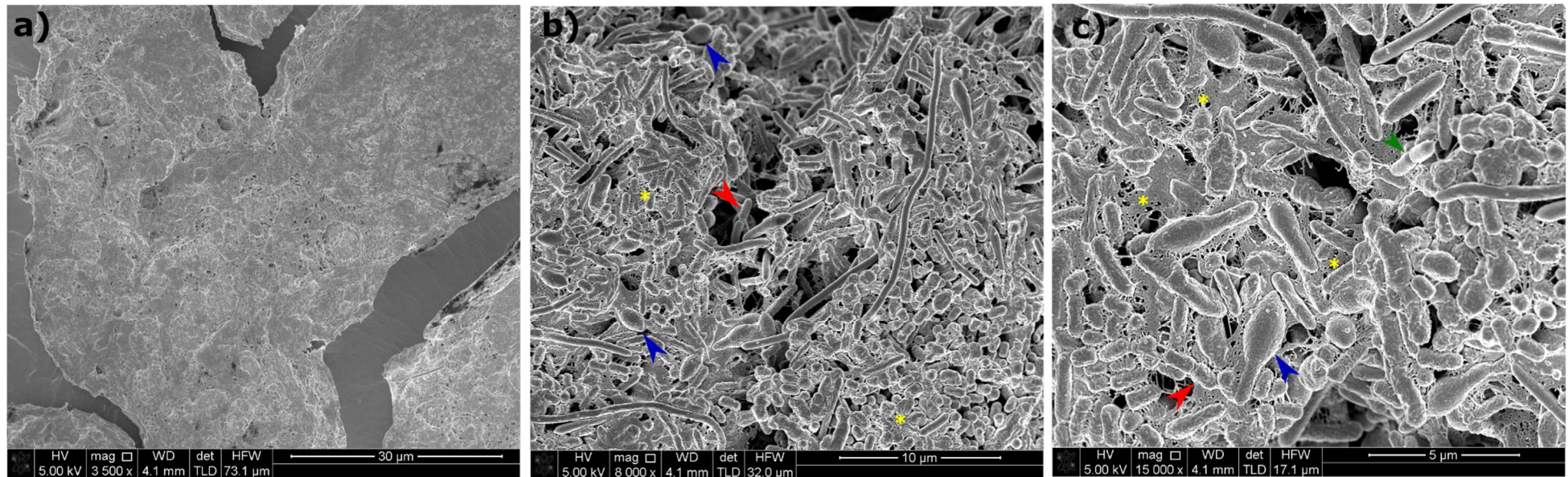
Figure 3-4: **Biofilm of extracted tracheoesophageal speech valve**
Scanning electron micrographs of TESV demonstrating a range of microorganisms such as yeast cells with germ tube (blue arrow), rods (red arrows) and surrounding with mesh-like biofilm matrix (yellow asterisk). Bacterial septa (green arrow) can be clearly seen in (c). (a) ×3500 magnification; (b) ×8000 magnification; (c) x15000 magnification.

3.3 Culture Independent Analysis

Isolation and identification of microorganisms from TESVs was performed using methods adapted from the NHS guideline for UK Standards for Microbiology Investigations for Sinusitis (Health-Protection-Agency, 2012). Culture independent analysis by next generation sequencing was used to determine the diversity of polymicrobial (bacterial and fungal) populations in TESV samples. Biofilm samples were obtained from 10 TESVs (TESV 7 – TESV 17, with the exception TESV 14 as this sample was contaminated during processing).

3.3.1 Optimization of DNA extraction

A DNA extraction technique was developed for simultaneous extraction of fungal and bacterial DNA. Initially two kits were tested; MasterPure™ DNA purification kit (Cat. No. MCD85201) (Cambio Ltd, Cambridge, UK) and ZR fungal/bacteria DNA miniprep (Cat. No. D6005) (Cambridge Bioscience, Cambridge UK). The kits were employed to extract DNA from stationary phase cultures of two *Candida* spp. (*C. tropicalis* SC4 and *C. albicans* SC10) and two Gram-positive bacteria, *S. salivarius* SB4 and *S. aureus* SF6. The concentrations of extracted DNA were measured using a NanoDrop® ND-1000 spectrophotometer (Table 3-1) and samples were analysed by AGE (Figure 3-5).

In samples extracted using MasterPure™ DNA purification, the concentrations of DNA were higher compared to those samples extracted by the ZR fungal/bacteria DNA miniprep kit (Table 3-1). By AGE analysis, the MasterPure™ DNA purification kit gave the highest yield of chromosomal DNA (thick band on the gel) but with a large amount of impurity present, which was probably RNA. By comparison, the ZR fungal/bacteria DNA miniprep kit produced visible single bands of DNA but at much lower DNA concentration. Therefore, a modified protocol was developed using components from both kits to enhance the efficiency for extraction of DNA from polymicrobial samples.

In the modified MasterPure™/ ZR DNA extraction the samples were washed with T&C lysis solution after treatment with lysozyme and mutanolysin. The samples were then incubated with proteinase K and RNAse (both from MasterPure™) in BashingBead™ Lysis (ZR DNA) tubes in accordance with manufacturer's

instructions (detailed method in Section 2.5.1). The result of using the modified technique was an increased DNA concentration over the ZR fungal/bacteria DNA miniprep with no low molecular weight nucleic acid contamination as shown in Figure 3-5. For example, the DNA concentration of *S. salivarius* SB4 was higher when using the modified method compared to the ZR fungal/bacteria DNA miniprep and exhibited only one thick band, in contrast to samples treated with the MasterPure™ DNA purification kit, which had an additional band migrating at low molecular weight. There was also an increase in product yield of *Candida* sp. (SC14) using the modified method when compared to just using ZR fungal/ bacteria DNA mini prep. Similar increases in DNA concentration with the combined method were observed in bacteria (SB4, SCH1, SCH14).

Sample ID	DNA concentration (ng/ µL)	A260/ A280 ratio	A260/A230 ratio
MasterPure™ DNA purification			
SC4	3047.7[a]	2.17	2.31
SC10	1495.7[a]	1.95	1.18
SB4	2352.7[a]	2.08	2.06
SF6	2872.3[a]	2.06	2.23
ZR fungal/bacteria DNA miniprep			
SC4	9.4	1.56	0.14
SC10	103.6	1.56	0.59
SB4	24.5	1.66	0.59
SF6	285.4	1.87	2.10
Modified MasterPure™/ ZR DNA extraction			
SCH1	719.2	1.85	2.05
SB4	306.7	1.9	1.9
SCH14	804.1	1.88	1.75
SC14	67.6	1.8	0.3

Table 3-1: **The DNA concentration of 4 different strains using two different kits independently.**
The strains used were *C. tropicalis* SC4, *C. albicans* SC10, SC14, *S. salivarius* SB4, *S. aureus* SF6, SCH1 and *L. paracasei* SCH14.[a] Nanodrop reading does not represent concentration of DNA only but also RNA.
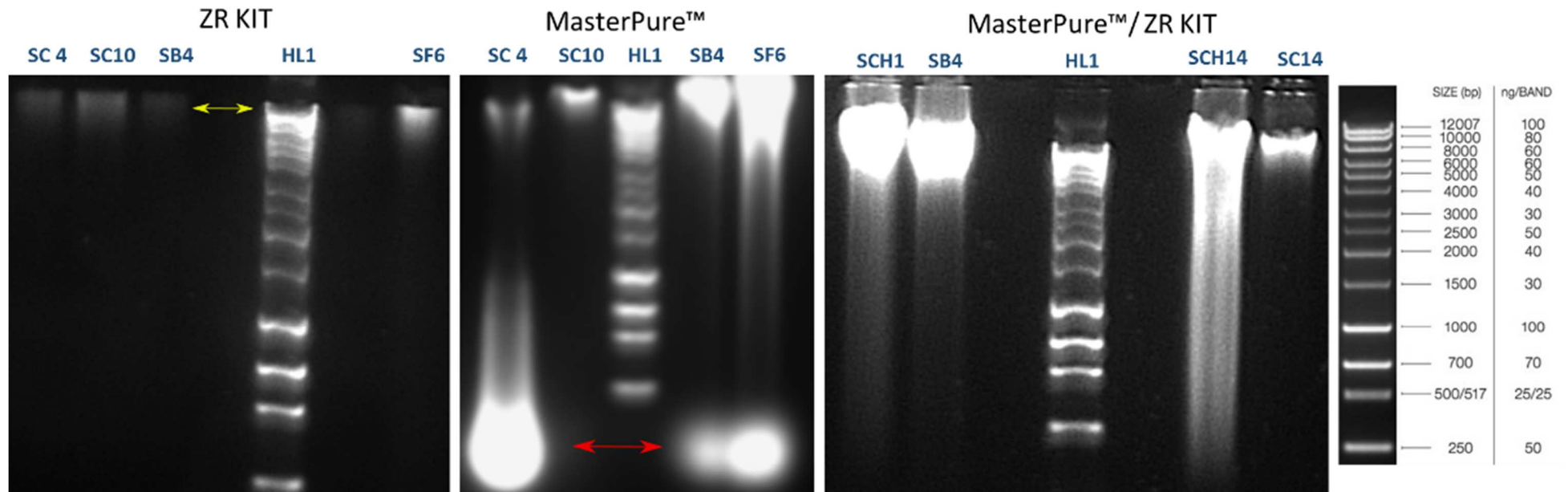
Figure 3-5: **The end products of DNA extraction using the ZR fungal/bacteria DNA miniprep, MasterPure™ DNA purification kit extraction or modified MasterPure™/ ZR fungal/bacteria DNA extraction.**
Yellow arrow highlights intact chromosomal DNA while the red arrow indicates putative RNA. HL1 is hyperladder 1.

3.3.2 Culture independent analysis of the TESV microbiota: Illumina® 16S/18S MiSeq™

Samples were extracted from 10 different TESVs using the modified extraction method and the DNA concentrations are shown in Table 3-2.

Sample Label	Concentration (ng/µl)
TESV 7	66.3
TESV 8	11.3
TESV 9	13
TESV 10	3.5
TESV 11	38.8
TESV 12	59.4
TESV 13	46.4
TESV 15	14.1
TESV 16	19.3
TESV 17	29.1

Table 3-2: **DNA concentration of 10 different TESV samples extracted using modified DNA extraction method.**

The total overall microbial populations on ten TESVs were assessed by sequencing PCR-amplified 16S or 18S fragments using Illumina® 16S/18S MiSeq™ and downstream bioinformatics analysis was performed by Scot E. Dowd (www.mrdnalab.com, Shallowater, TX, USA). Analysis of bioinformatics data, showed that *Candida* spp. were dominant among the fungi in all ten TESVs (refer to Figure 3-6). *Saccharomycetes* were identified in 9 out of 10 samples while *Nakaseomyces* (e.g. *C. glabrata*) was present at >1% of the total fungal population in TESV 11 and 16.

The bacterial composition of TESVs consisted of both Gram positive and Gram-negative bacteria (Figure 3-7). The genus *Lactobacillus* (10/10 TESVs) was the most commonly identified in the biofilms followed by *Staphylococcus* (8/10 TESVs). Oral related genera such as *Streptococcus*, *Veillonella*, *Rothia*, *Prevotella*, *Actinomyces* and *Gemella* were present at lower frequencies.
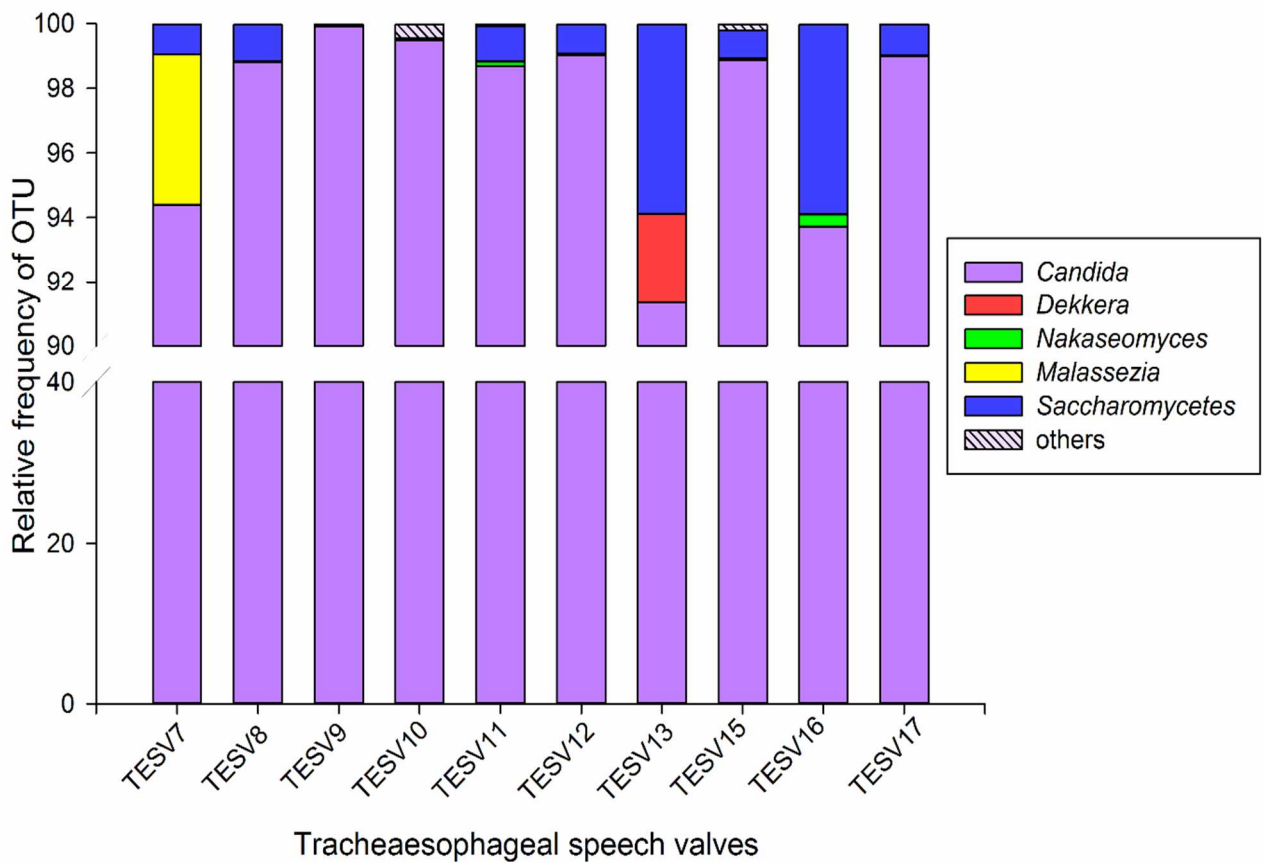
Figure 3-6: **Culture independent analysis of Eukaryota communities of extracted TESVs.**
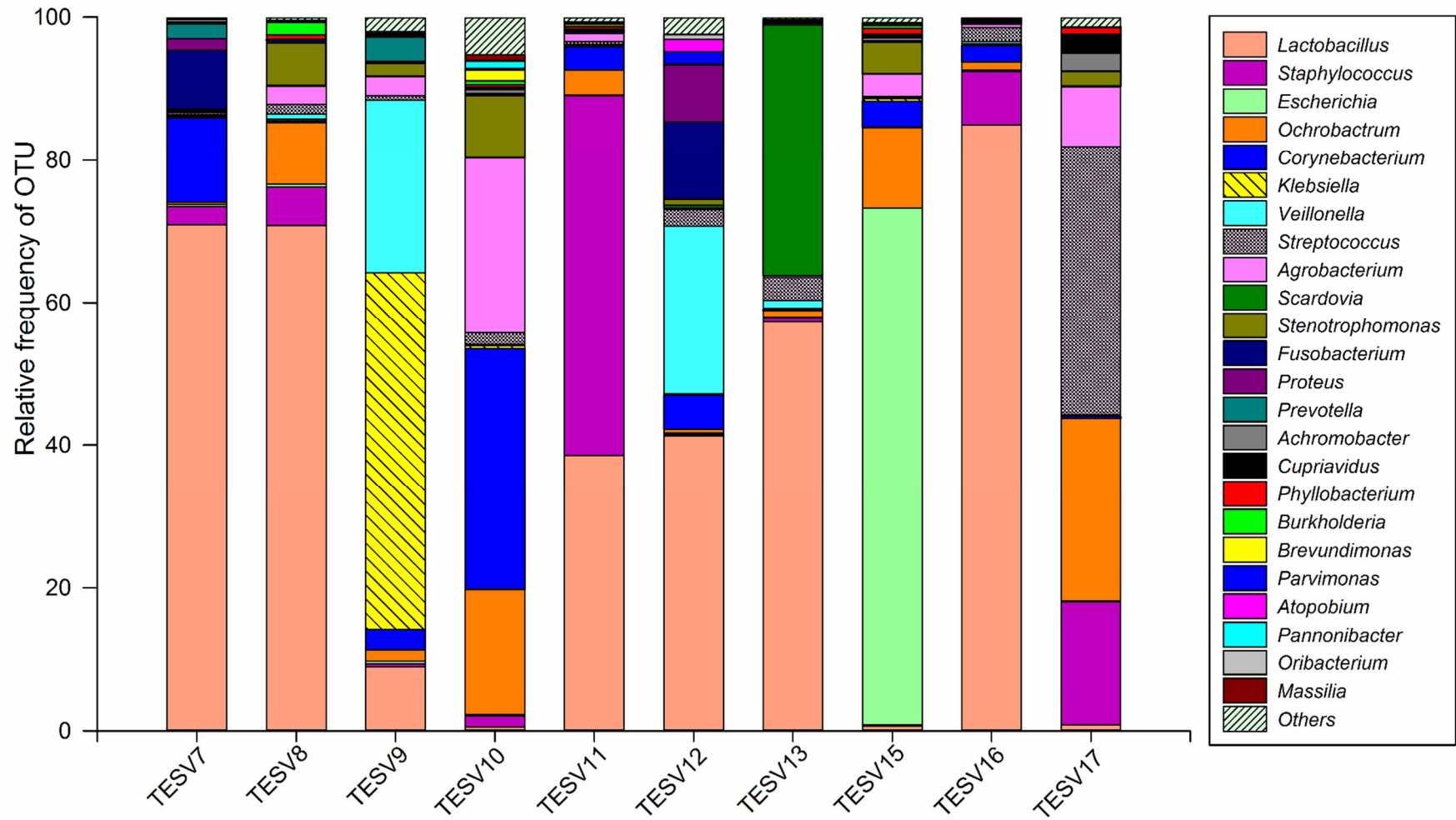
Figure 3-7: **Culture independent analysis of bacteria from extracted TESVs**.

3.4   Identification of TESV Biofilm Community Members

Culture of a broad range of species was attempted using a variety of growth media such as blood, chocolate, fastidious anaerobic and MRS agars. The species that grew were screened for phenotypic properties such as colony morphology, Gram staining and cell morphology. Selective and indicator agars such as Staph agar and CHROMagar™ for *Candida* spp. (Figure 3-8) were also used.

Figure 3-8: **Identified *Candida* spp. were tested on CHROMagar™ for *Candida* spp. differentiation.**
Blue colonies represent *C. albicans* NU14; pink, *C. tropicalis* NU36 and white, *C. glabrata* NU15. Other types of *Candida* spp. would also appear as white colonies on this medium.

3.4.1 Isolation

Sonicated liquid samples were spread on different agar media thinly and incubated at 37 °C for 96 h in either an anaerobic chamber or a benchtop incubator. From observation, the biofilm samples produced confluent growth. Numbers of colonies were highest on blood and chocolate agar cultured aerobically compared to other media such as MRSA and SD agar, or on FAA cultured anaerobically. Both chocolate and blood media are nutrient-rich media and provide a range of nutritional components such as haem, minerals, and proteins.

Colonies were isolated according to their appearance, morphology and colour and inoculated on fresh agar media. This was important to obtain pure samples and prevent contamination. The colonies were then screened for morphology using Gram staining (refer to Section 2.3.1) and various phenotypic characteristics including hydrogen peroxide (H_2O_2) production, extracellular DNase activity and catalase production for preliminary identification (refer to Section 2.4).

3.4.2 Screening of biofilm community

Biochemical testing was performed to screen for the production of DNase, catalase and H_2O_2. The findings of community screening are shown in Table 3-3. After the screening, the samples were transferred to the Pathology Unit, Freeman Hospital where MALDI-TOF MS identification was performed.

Strain[a]	Gram stain	Environment requirement/ colony colour	DNase test	H_2O_2	Catalase
Candida albicans (11)	N/A	bluish colony on ChromID	ND	-	+
Candida glabrata (3)	N/A	whitish colony on ChromID	ND	-	+
Candida tropicalis (5)	N/A	pink colony on ChromID	ND	-	+
Ochrobactrum sp. *(5)*	G- rod	obligate aerobic	ND	-	+
Staphylococcus aureus (8)	G+ cocci	aerobic, yellow colony on staph agar	+	-	+
Staphylococcus epidermidis (2)	G+ cocci	aerobic	-	-	+
Klebsiella sp. *(5)*	G- bacilli	aerobic	ND	-	+
Lactobacillus spp. *(20)*	G+ bacilli	anaerobic	-	+	-
Streptococcus spp. *(8)*	G+ cocci	facultative aerobic	+	+[b]	-

Table 3-3: **Characteristics of strains isolated from TESVs.**
[a] Number in parentheses represents the quantity of isolated species. +[b]Some of the *Streptococcus* spp, did not produce H_2O_2;(more detail in Chapter 4) +: detected; -: not detected; ND: not done

3.4.3 Germ tube formation by Candida spp.

Germ tube formation is a unique morphological change observed in certain *Candida* spp. such as *C. albicans* and *C. tropicalis* but not *C. glabrata*. Overnight *C. albicans* NU33, NU14, NU35, SC3415 and *C. glabrata* NU35 cultures were diluted 1:10 and incubated at 37 °C for up to 6 h whilst shaking (180 *rpm*) in the presence of 10% fetal calf serum to induce germ tube formation. The cellular morphology was visualised by differential interference contrast (DIC) microscopy.

Haploid *C. glabrata* were classified as non-forming hyphae *Candida* sp. as incubation with serum did not trigger any such morphological changes (Figure 3-9). Meanwhile in *C. albicans*, all strains including wild-type *C. albicans* (SC3415) formed germ tubes after one-hour incubation with fetal calf serum (Figure 3-10). After 4 h, hyphae production was more extensive, and they were all entangled.
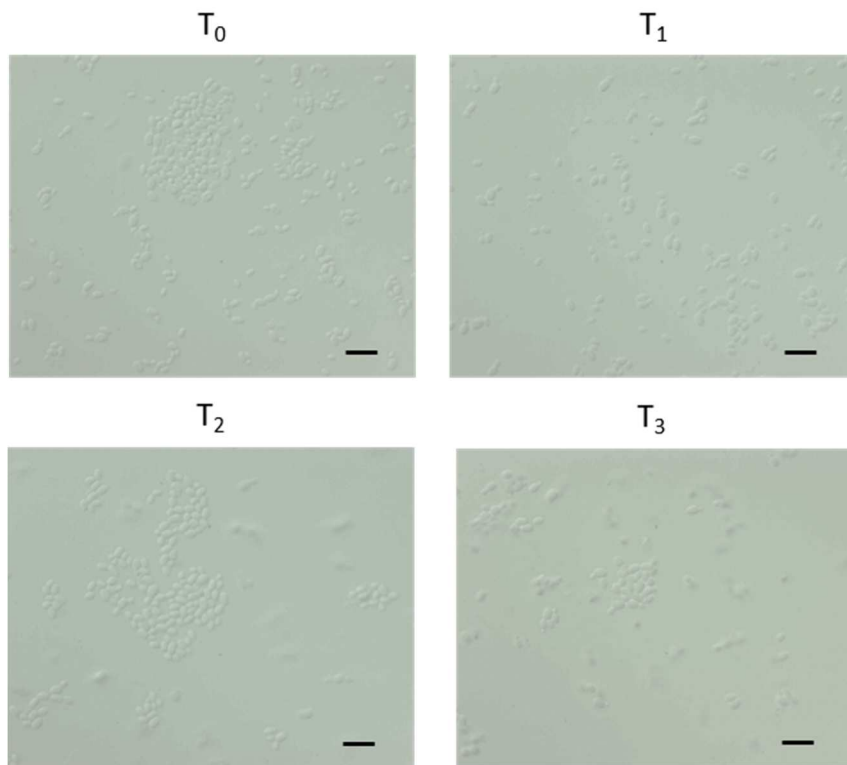
Figure 3-9: *C. glabrata* **NU34 yeast cells after the exposure of fetal calf serum.** Scale bar 10 µm.
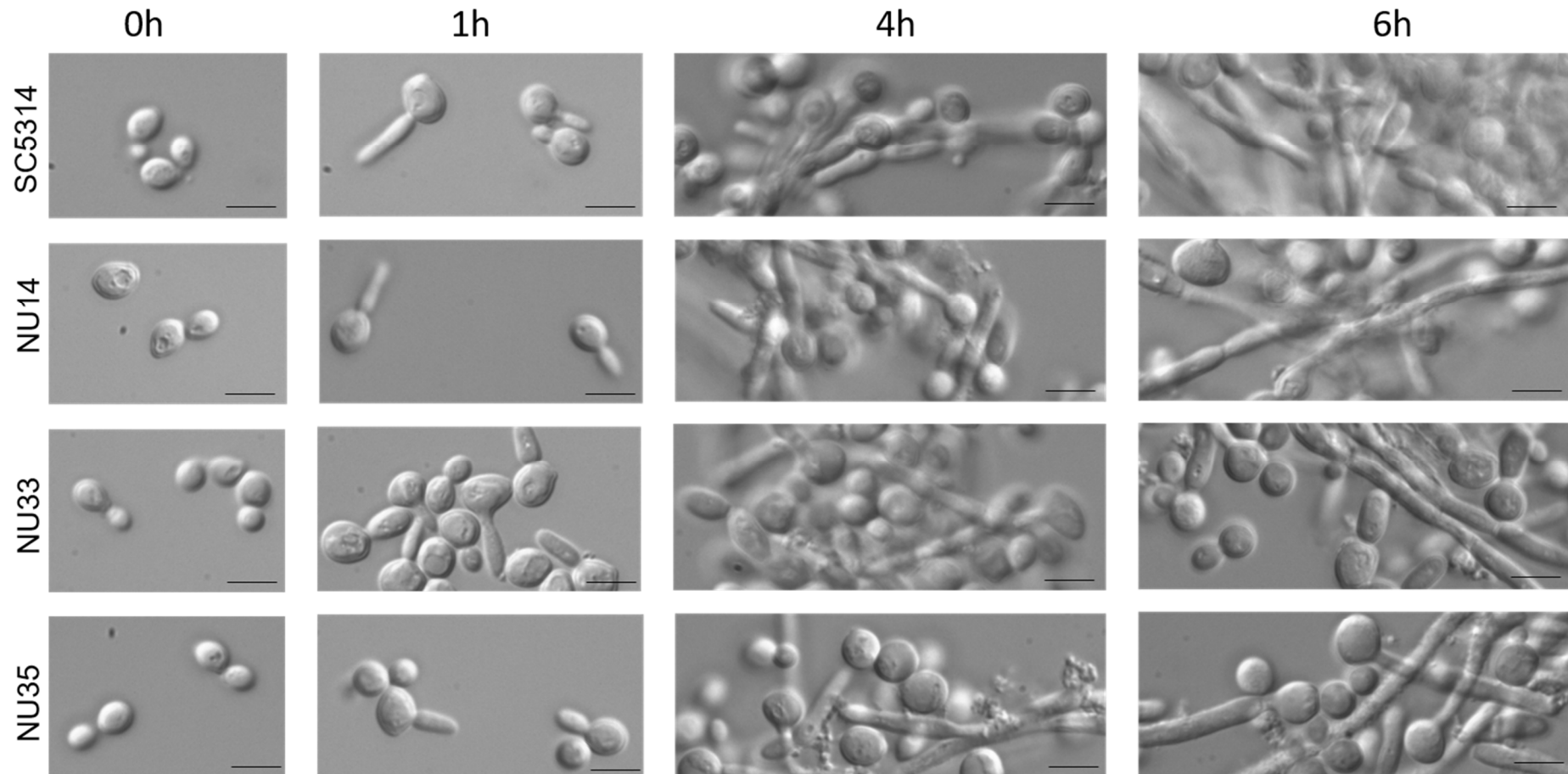
Figure 3-10: **Comparison of morphological switching in clinical isolates of *C. albicans* compared to the SC5314 reference strain.** All *C. albicans* strains tested switch from yeast cells (time zero), to form germ tubes (after an hour) and subsequently hyphae, after serum treatment at 37°C for 4-6 h. Scale bar 10 μm.
.

3.4.4 Species identification using MALDI-TOF MS

Following isolation and screening of microbial communities from clinical samples, strains were sent for identification using culture dependent MALDI-TOF MS. Clinical isolates from 16 TESVs were analysed (Table 3-3) and identified as shown in Table 3-4.

The most frequently isolated fungal species was *C. albicans*, which was cultured from 11 out of 16 TESVs (79%), followed by five TESVs with *C. tropicalis* (36%), three TESVs had *C. glabrata* (21%) and only one TESV contained *S. cerevisiae* (7%). Interestingly no biofilm communities contained more than two fungal species and 2 TESVs (12%) possessed only bacterial species.

Species from the genus *Lactobacillus* were found in 10 of 16 TESVs (63%), the highest frequency of any bacterial genus isolated from TESVs. *L. fermentum* were isolated from seven TESVs, followed by *L. paracasei* (6), *L. rhamnosus* (3), *L. plantarum* (3) and *L. gasseri* (1). Another lactic acid producer, *P. pentasaceus,* was isolated from one of the valves.

There were four *Streptococcus* spp.: five TESVs contained *S. salivarius* and one contained *S. anginosus*, *S. oralis* and *S. parasanguinis*. All *Streptococcus* species were identified as normal flora of the oral cavity and throat.

S. aureus was the most frequently isolated bacterial species, found in eight TESVs of 16. *S. epidermidis,* commonly a member of the normal flora of skin was identified in two TESVs (13%). *K. oxytoca* and *O. anthropi* were both identified in five different TESVs, while the gut bacterium *E. faecium* was found in four TESVs. Only one TESV contained *E. coli*.

Species	Tracheaesophageal speech valve															
	1	2	3	4	5	6	7	8	9	10	11	12	13	15	16	17
Fungi																
Candida albicans				x	x	x	x	x			x	x	x	x	x	x
Candida glabrata						x					x				x	
Candida tropicalis	x					x	x	x	x							
Saccharomyces cerevisiae														x		
Bacteria																
Streptococcus anginosus												x				
Streptococcus salivarius	x	x	x			x		x								
Streptococcus oralis															x	
Streptococcus parasanguinis	x															
Staphylococcus epidermidis											x				x	
Staphylococcus aureus	x	x	x		x			x			x				x	x
Lactobacillus paracasei				x	x	x		x	x			x				
Lactobacillus fermentum				x	x	x	x				x	x			x	
Lactobacillus rhamnosus				x								x	x			
Lactobacillus gasseri						x										
Lactobacillus plantarum					x	x	x									
Klebsiella oxytoca		x	x	x				x			x					
Enterococcus faecium		x	x	x												x
Orchrobactrium anthropi									x	x	x				x	x
Escherichia coli														x		
Pediococcus pentasaceus						x										

Table 3-4: **Identification of species isolated from 16 used TESVs by MALDI-TOF MS.**
A MALDI Bio-typer software was used for species identification in accordance with manufacturer's instruction.
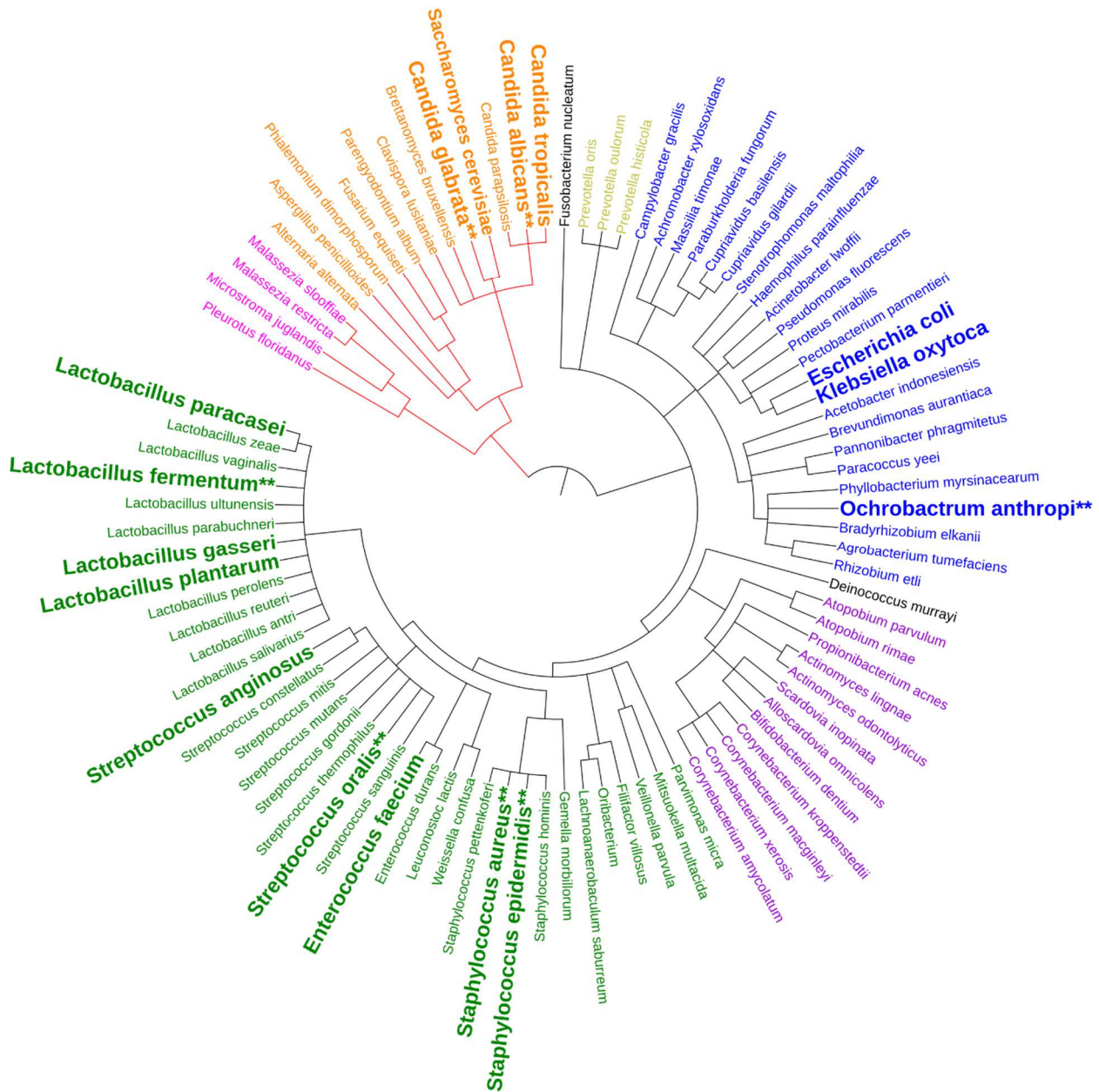
Figure 3-11: **Phylogenetic tree showed 10 TESVs sequenced communities**. The red branches representing eukaryota and the black is for bacterial species. Microbial species in bold represent species that were isolated in the lab and ** is for samples that isolated and used in this project. Bacterial species from Firmicutes are highlighted in green, Actinobacteria (purple), Proteobacteria (blue), Bacteroidetes (yellow), and Fusobacteria (black) while in fungi are saccharomyceta in orange and basidiomycota (pink) respectively. http://phylot.biobyte.de/ (Letunic and Bork, 2016)

3.5   Discussion

Biofilms were clearly observed on the silicone surfaces of all failed TESVs analysed in this study. Cultivation of biofilm samples was done between 96 to 240 h which was selected as an appropriate range for clinically isolated microflora (Pratten *et al.*, 2003). Recently, culturomics terminology has been introduced by Lagier *et al.* (2015). This approach consists of culture dependent traditional microbiology techniques, MALDI-TOF MS and the application of high-throughput culture independent methods. The culture independent technique was used to study human microbiota using 16S/18S rRNA amplification and NGS sequencing for the identification of cultured colonies, and previously unidentified (using culture dependent technique) species (Lagier *et al.*, 2015). MALDI-TOF MS is routinely used in many clinical microbiology labs, however, this culture dependent technique requires pure cultures and may take up to 24 h for identification. The results showed that MALDI-TOF MS data alone could not accurately represent the whole biofilm community as there are bacterial species that cannot be cultured by traditional techniques. Indeed, it has been estimated that less than 2% of the bacteria on Earth can be cultured using artificial media (Wade, 2002). Nevertheless, MALDI-TOF MS is considered a suitable technique in routine bacterial identification as it is rapid and cost effective (Lagier *et al.*, 2015; Navrátilová *et al.*, 2016).

The same approach was taken into consideration while handling and identifying TESVs samples in this study. A modified DNA extraction technique was used to gain the highest yield while collecting samples from the TESVs that a good method is needed to maximise the prospects of detecting rare species (Kennedy *et al.*, 2014). In the modified method, there were a few steps and chemicals obtained from the MasterPure™ DNA purification kit. This kit is commonly used in our laboratory to extract DNA from pure culture samples. Samples were incubated with proteinase K and RNAse to eliminate RNA contamination. As samples from TESV biofilm composition are commonly a mixture of bacterial and fungal cells, ZR fungal/bacteria DNA miniprep steps and chemicals were incorporated into the modified method. The cell lysis of the samples was facilitated by using BashingBead™ Lysis tube and Zymo-Spin™ IIC column helped to concentrate the samples. This modified technique required only an hour to extract DNA compared to more than 4 h needed when using the MasterPure™ DNA purification kit only. The optimization of the DNA extraction

method was crucial (Henderson *et al.*, 2013) due to the limited amount of biofilm material in the TESV samples and to avoid any DNA degradation during sample handling.

The location between the trachea and oesophagus and exposure to the outside environment via a stoma provides an environment that supports the growth of the rich polymicrobial TESV community. A phylogenetic tree was mapped from our findings and appears in Figure 3-11, indicating the mixture of both fungal and bacterial species isolated and identified using the culturomics approach. There are many different bacterial species such as *Bifidobacterium infantis*, *Enterococcus faecium*, *Lactobacillus* spp. and *S. thermophilus* that have been isolated from TESVs (Van Der Mei *et al.*, 2000; Tićac *et al.*, 2010). Oral microbial species were also identified and contributed to the formation of complex communities in TESV biofilms (Tićac *et al.*, 2010; Holmes *et al.*, 2012; Somogyi-Ganss *et al.*, 2017).

The screening of communities using different biochemical tests is important to prepare samples for MALDI-TOF MS and give some level of validation. Lactic acid bacteria are highly associated with fungi in polymicrobial biofilms in many medical devices (Buijssen *et al.*, 2012; Martinez *et al.*, 2016; O'Donnell *et al.*, 2016). Our analysis showed that *Lactobacillus* spp. and *Streptococcus* spp. were most commonly identified in TESV biofilms. These Gram-positive bacteria were the only species that produced H_2O_2 while three species of bacteria (*Ochrobactrum* sp*.*, *Staphylococcus* sp*.* and *Klebsiella* sp.) and *Candida* sp*.* produced catalase. This information is important as there are studies that have linked the effect of H_2O_2 to the mixed fungal bacterial biofilm formation (Boris and Barbes, 2000; Strus *et al.*, 2005)

H_2O_2 is known as one of environmental stressors in *C. albicans* that causes cell cycle arrest which induces a different filamentous form (hyperpolarised buds) (da Silva Dantas *et al.*, 2010) which may promote fungal biofilm formation. The plasticity of *C. albicans* interchanging its morphology according to environmental signals establishes it as important player in the creation of a robust biofilm (Soll and Daniels, 2016). This trait enables colonization of tissues and adherence to surfaces (Saville *et al.*, 2003). The clinically isolated *C. albicans* were able to form hyphae similar to the reference strain (refer to Section 3.4.3), and provides a platform for further investigations into the effects of environmental stresses on the yeast/hyphal transition in such isolates. This is important as several reports point out that hyphae

formation has a significant effect on the establishing of mixed species biofilms (Diaz *et al.*, 2012b; Nobbs and Jenkinson, 2015). There is also a need to incorporate other hyphae-forming fungi such as *C. tropicalis* into the biofilm model as both were also identified in medical device biofilms.

There were 91 species identified using NGS compared to only 16 species from culture and MALDI-TOF MS. Many are bacterial species that can also be found in different anatomical locations such as the oral cavity (*S. oralis and S. salivarius*), gut (*E. faecium, Klebsiella* spp. and *E. coli*) and on the skin (*S. aureus and S. epidermidis*) (Leonhard *et al.*, 2010). *Fusobacterium* spp. were also detected in the sequencing analysis which agrees with the study by Bertl *et al.* (2012) that reported the existence of anaerobic and microaerophilic oral pathogens such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* on TESVs. Firmicutes was the most frequently identified and isolated bacteria phylum in the laboratory setting (shown in phylogenetic tree Figure 3-11). The most frequent microbes were lactic acid bacteria such as lactobacilli and streptococci which have been reported by several studies (Van Der Mei *et al.*, 2000; Tićac *et al.*, 2010).

From the NGS sequencing data, *C. albicans* was detected in all TESVs (Figure 3-6). Different types of fungi were also found in the community such as non-albicans *Candida* (including *C. krusei*, *C. tropicalis*, *C. glabrata* (Nakaseomyces) and *C. parapsilosis*), *Saccharomyces cerevisiae* and *Pichia etchellsii*. This is consistent with previous culture independent analyses that showed, not only *Candida* spp. (Leonhard *et al.*, 2010; Tićac *et al.*, 2010; Holmes *et al.*, 2012; Somogyi-Ganss *et al.*, 2017), but also *Saccharomycetes*, *Dekkera* and *Malassezia* were sequenced from TESVs samples (Neu *et al.*, 1994b). However, only *Saccharomyces cerevisiae* (TESV 15), *C. glabrata* (TESV 6, 11 and 16) and *C. tropicalis* (TESV 7, 8, 9 and 10) were isolated (See Table 3-4) as it is difficult to culture many fungi (e.g. *Malessezia*).

The mesh-like structures seen in Figure 3-4 are similar to those formed by eDNA in *E. faecalis* biofilms (Barnes *et al.*, 2012) and in subgingival biofilm (Holliday *et al.*, 2015; Rostami *et al.*, 2017). *C. albicans* were also reported to release extracellular DNA in a strain dependent fashion and is associated with biofilm heterogeneity (Rajendran *et al.*, 2014). Therefore, it is possible that eDNA is an important part of the matrix of TESV biofilms as there was evidence indicating that eDNA is present in these biofilms (Shakir *et al.*, 2012). A number of DNAse producers (refer to Table

3-3) were isolated in the polymicrobial TESV biofilm. It is important that this characteristic should be studied for TESVs biofilm in the future as there is potential to target eDNA for biofilm control.

Chapter 4. Characterisation of tracheoesophageal speech valve microbial isolates

4.1 Introduction

The changes in numbers and proportions of individual members within a biofilm community as it develops over time is termed microbial succession. These changes are influenced by numerous factors that include the surrounding environment and the other biofilm members. Key environmental variables such as pH, availability of nutrients and redox potential play a role in determining the types of species that can co-exist within the biofilm community. Interestingly, as shown in the previous chapter, TESV biofilm communities usually consist of a mixture of fungal and bacterial species. Recent studies are beginning to unravel the complex interactions between bacteria and *Candida,* reporting a variety of endogenous and exogenous environmental stresses that strongly affect the biofilm community dynamic (Förster *et al.*, 2016; Gambino and Cappitelli, 2016).

Influences such as the presence of antimicrobial agents, specific ions and oxidative stress can all determine the outcome of the microbial competition in complex biofilm communities. Many studies have investigated the impact of oxidative stress, salts, metal ions, or a combination of stress exposures on *C. albicans* (Yin *et al.*, 2009; Kaloriti *et al.*, 2014) (reviewed by (Brown *et al.*, 2014)) and/or on different bacteria (reviewed in Grant and Hung (2013)). These stresses can trigger various gene regulatory responses in cells that may contribute to the development of a stress-resistant phenotype (Brown *et al.*, 2009; Dantas *et al.*, 2015).

Reactive oxygen species (ROS) are commonly generated within the biofilm environment (reviewed in Čáp *et al.* (2012)). An accumulation of ROS contributes to oxidative stress where growth of susceptible microbial cells can be impaired. H_2O_2 is an important reactive oxygen molecule that reacts with DNA and other critical cellular components and can lead to significant cell damage and ultimately cell death (Cabiscol *et al.*, 2000). *Lactobacillus* spp. release H_2O_2, lactic acid and biosurfactants (Rodrigues *et al.*, 2004) which can inhibit *C. albicans* proliferation and invasive hyphal formation (Boris and Barbes, 2000). Microbial species protect themselves in order to prevent damage from the adverse effects of H_2O_2 by the

induction of enzymes, such as peroxidase and catalase, to degrade H_2O_2, thus eliminating the potential oxidative damage caused by this chemical assault. Community members lacking the ability to produce these enzymes can also benefit from their neighbours' oxidative stress enzymes that reduce the local H_2O_2 concentration (Jakubovics *et al.*, 2008b). However, while elevated levels of ROS can be toxic, lower levels can stimulate cells to employ specific scavenging systems or alter metabolic pathways in order to survive. For example, the production of H_2O_2 by early oral biofilm colonizers, such as oral streptococci, is an important protection mechanism against competing species (Jakubovics *et al.*, 2008b).

In addition to chemical influences, physical interactions occur that can be either mutually beneficial or create competition between co-existent microorganisms in the same community. These can be either coaggregation (between different species) or auto-aggregation (by the same species) (Katharios-Lanwermeyer *et al.*, 2014). Specific adhesins on cell surfaces can recognize and bind to receptors on neighbouring cells, so the cell surfaces of member species dictate coaggregation interactions amongst the biofilm community (Kolenbrander *et al.*, 2002). These interactions are considered to be a survival mechanism (Slavkin, 1997; Bowden and Hamilton, 1998) and are also important for nutrient supply and the exchange of signalling molecules between species (Kolenbrander *et al.*, 2010).

Coaggregation has been studied extensively with oral bacteria (Kolenbrander, 2000) (Bamford *et al.*, 2009; Arzmi *et al.*, 2015; Nobbs and Jenkinson, 2015; Stevens *et al.*, 2015; Palmer *et al.*, 2017). Subsequently, in more recent years, coaggregation within biofilm communities isolated from a variety of locations in the human body, from freshwater and from food have also been reported (Wingender and Flemming, 2011; Stevens *et al.*, 2015). The physical binding between taxonomically distinct species isolated from discrete environments was shown to be possible and species that can undergo such interactions were labelled 'cross-environment co-aggregating organisms' (Stevens *et al.*, 2015). This ability has been suggested to aid the colonization of non-indigenous species in new biofilm community environments.

Coaggregation is important in biofilm formation as the community will arrange cellular mosaics, which facilitate cell-cell signalling and enable metabolic cross-feeding to form an organized structure that enhances the persistence of biofilm and preserves the individual member cells (Davey and O'toole, 2000; Kolenbrander *et al.*,

2010). This property also influences the specificity of adhesion which significantly contributes to the succession of biofilm development through its various stages, from initial colonisation to maturity (Hojo *et al.*, 2009). These cell specific binding interactions are important in biofilm formation (Rickard *et al.*, 2003) and are considered to be a virulence trait in pathogenic biofilms (Sato and Nakazawa, 2014). As mentioned previously, microbial species obtained from different environments such as the oral cavity, aquatic biofilm and human nasopharynx are able to coaggregate with each other (Stevens *et al.*, 2015). It is important to study the possible cell-cell interactions between species isolated from TESV biofilms to identify which species specifically bind to one another. An increased understanding of the different properties of the fungal and bacterial community members in such biofilms may potentially lead to improved methods for biofilm management.

The study set out to characterise the key physical and chemical interactions between members of selected TESV biofilms. The specific objectives here were (1) to identify species to be used for an *in-vitro* static biofilm model; (2) to monitor H_2O_2 production by bacteria; (3) to investigate and visualize coaggregation interactions between isolates from a TESV and; (4) to assess the effect of environmental stressors on clinically isolated *Candida* spp.

4.2   Confirmation and Further Identification of TESV Isolated Strains.

The members of TESV biofilm communities were initially identified on the basis of morphology, simple phenotypic tests and MADLI-TOF MS as discussed in Chapter 3. In this chapter, we were aiming towards developing a new model containing strains that were naturally found together. In order to achieve this, the communities and their interactions needed to be characterised in more detail. Analysis of NGS data of TESV 8, 11 and 16 was performed to the species level and the results are shown in Figure 4-1 and Figure 4-2. These TESV samples were selected as we were able to isolate a similar composition of biofilm members that are representative of a TESV community in an *in vitro* static biofilm model.

The NGS data (Figure 4-1) showed all three TESVs contained *C. albicans*, *C. tropicalis* and *Saccharomycetes* spp. Other species detected were *C. glabrata* on TESV 11 and TESV 16 and *C. parapsilosis* was identified in TESV 11 only. Traces of

other species of fungi were detected on the TESVs such as *Malassezia slooffiae* and *Saccharomyces cerevisiae*.
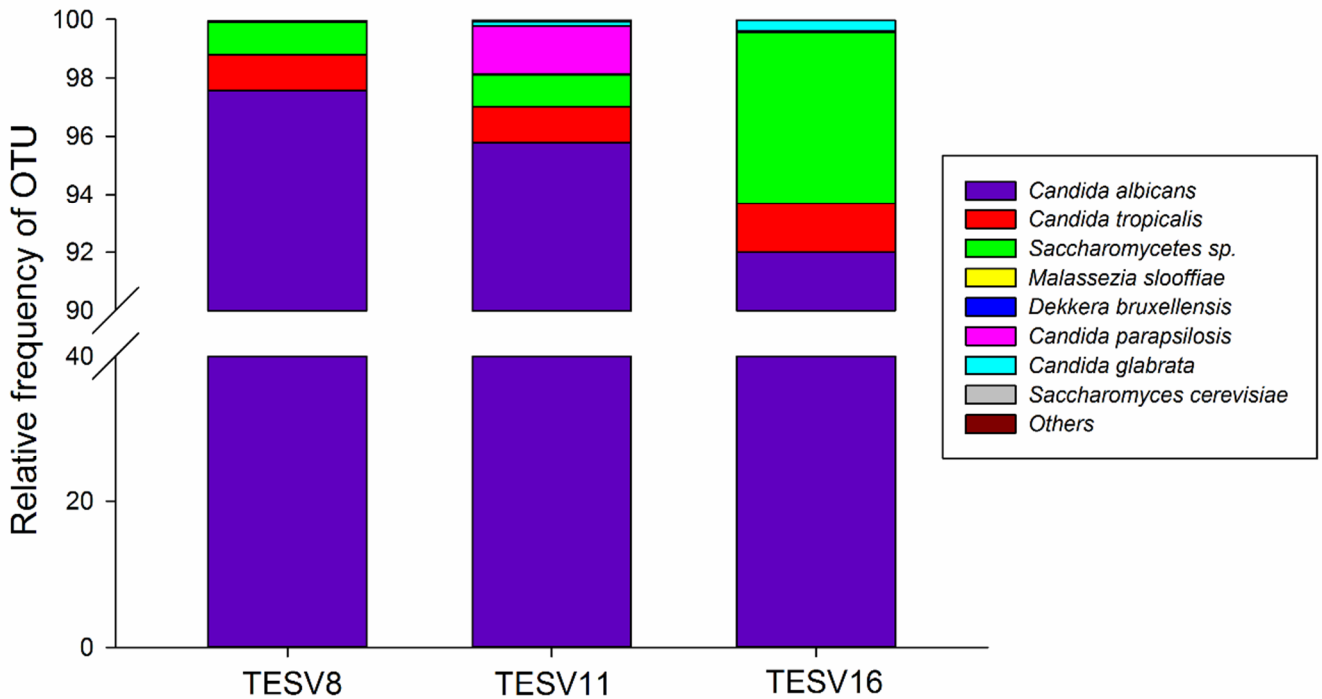
Figure 4-1: **The column chart represents fungi at the species level analysed from NGS data of TESV 8, TESV 11 and TESV 16.**
Only OTUs (clusters of similar sequence variants of the 16S/ 18S rDNA marker gene sequence that are defined by a 97% identity threshold at genus level) that constituted ≥0.1% of the total population were included.

Besides *Candida* spp., the eukaryotes *Phialemonium* sp. and *Saccharomycetes* sp. were detected but these species were not cultured in the laboratory. *C. albicans* was the most abundant identified eukaryotic species overall followed by *C. tropicalis* and then *C. glabrata*. Data from Figure 4-2 can be compared with the data in Table 4-1 which shows that of the bacterial species, *L. gasseri* was represented in all three TESVs and in the highest proportions (but not isolated in the laboratory) followed by the combination of *S. aureus* and *L. fermentum*. Both species were identified and isolated in all three TESVs
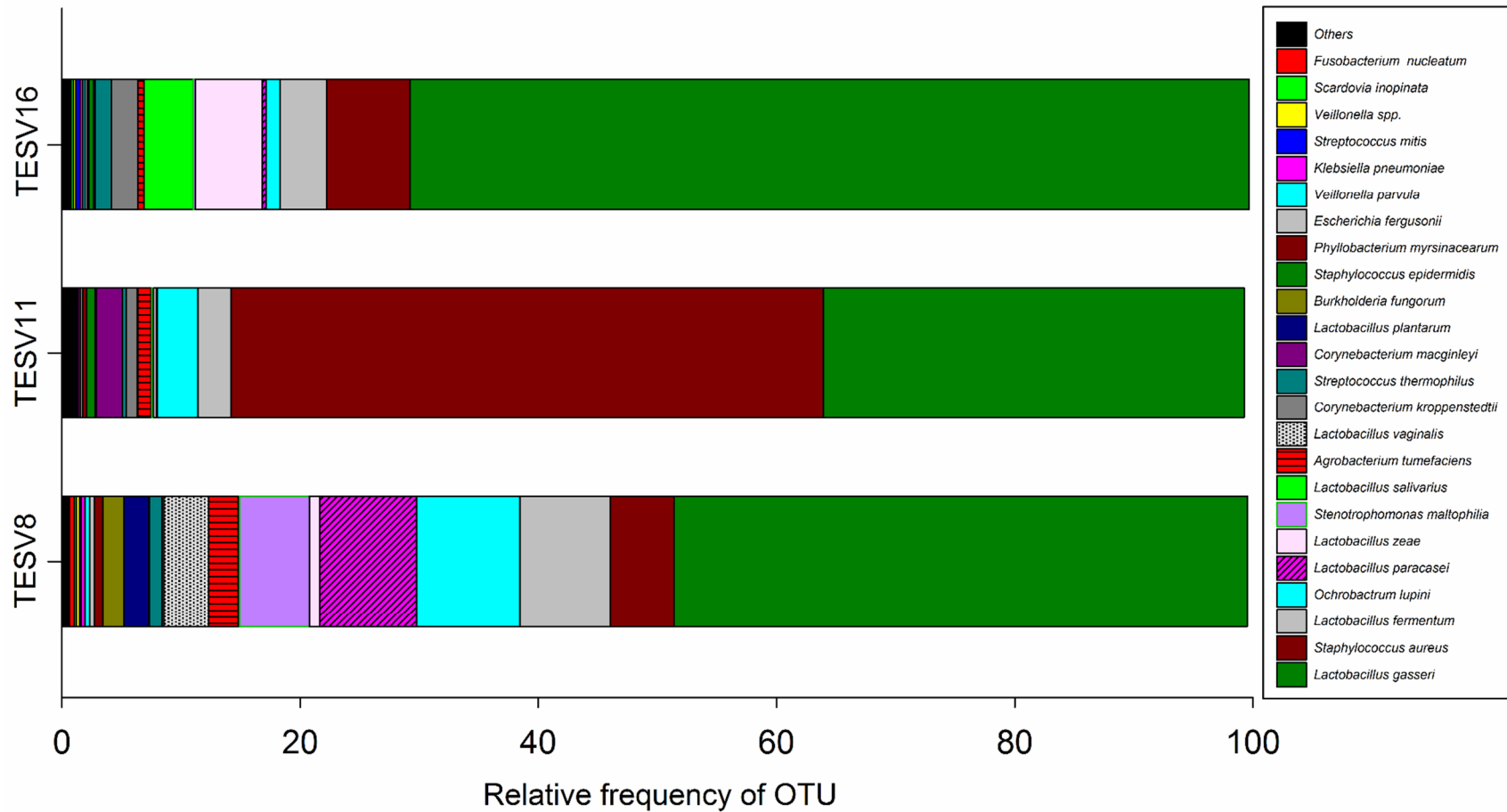
Figure 4-2: **The proportion of 16S rRNA OTUs represented by cultures increases with their relative abundance in TESV communities.**
The bar chart represents bacterial species analysed from NGS data of TESV 8, TESV 11 and TESV 16.

The bacterial species, *L. gasseri*, *S. aureus, Ochrobactrum* sp. and *L. fermentum* were major components of all three TESV biofilms. The species (identified by MALDI-TOF MS and PCR) and features of communities isolated from the three TESVs are tabulated and summarized in Table 4-1.

The isolated species were divided into catalase producing species; *C. albicans*, *C. glabrata*, *S. aureus S. epidermidis* and *O. anthropi* and H_2O_2 releasing bacteria; *L. paracasei*, *L. fermentum* and *S. oralis*. It is apparent from Table 4-1 that the biofilms isolated from TESV11 and TESV16 are similar in composition to one another and slightly different from TESV8. All three TESV biofilms contained *C. albicans*, *S. aureus* and *L. fermentum*. *C. glabrata*, *S. epidermidis* and *O. anthropi* were identified in two of the TESVs. The TESV 16 community was selected for further study as a representative biofilm as it possessed all six of these species in addition to *S. oralis*.

Isolated colonies were sent for identification using MALDI-TOF MS and in some cases identification was confirmed by PCR using universal 16S rRNA primers 63f and 1387r (Marchesi *et al.*, 1998) and DNA sequencing (refer to Table 4-2). Examples of PCR amplification products are shown in Figure 4-3. 16S rRNA gene sequencing was important as some of MALDI-TOF MS identification was only classified as 'high confidence' up to the genus level. In this case, *Ochrobactrum* spp. was identified by MALDI-TOF MS and by using 16S rRNA PCR, the sample was matched with *Ochrobactrum anthropi*.

Species	H$_2$O$_2$/ catalase producer	Tracheoesophageal speech valve		
		8	11	16
Fungi				
Candida albicans	Catalase	NU35	NU14	NU33
Candida glabrata	Catalase	-	NU15	NU34
Candida tropicalis	Catalase	NU36	-	-
Bacteria				
Streptococcus salivarius	X	NU32	-	-
Streptococcus oralis	H$_2$O$_2$	-	-	NU31
Staphylococcus epidermidis	Catalase	-	NU29	NU30
Staphylococcus aureus	Catalase	NU28	NU26	NU27
Lactobacillus paracasei	H$_2$O$_2$	NU22	-	-
Lactobacillus fermentum	H$_2$O$_2$	NU21	-	NU19
Klebsiella oxytoca	Catalase	-	NU16	-
Ochrobactrum anthropi	Catalase	-	NU24	NU25

Table 4-1: **Tabulation of the species and strains isolated from TESV 8, TESV 11 and TESV 16.**
NU' numbers refer to the strain isolated from each valve; X catalase negative and does not produce H$_2$O$_2$.

Strain ID	ID by MALDI-TOF	PCR Product (bp)	ID by PCR
NU32	*Streptococcus salivarius*	1,300 bp	*S. salivarius*
NU31	*Streptococcus oralis*	1,300 bp	*S. oralis*
NU29	*Staphylococcus epidermidis*	1,300 bp	*S. epidermidis*
NU28	*Staphylococcus aureus*	1,300 bp	*S. aureus*
NU22	*Lactobacillus paracasei*	1,300 bp	*L. paracasei*
NU21	*Lactobacillus fermentum*	1,300 bp	*L. fermentum*
NU16	*Klebsiella oxytoca*	1,300 bp	*K. oxytoca*
NU24	*Ochrobactrum* sp.	1,300 bp	*O. anthropi*

Table 4-2: **Identification of isolated bacterial strains using 16S rRNA PCR and MALDI-TOF MS.**
The samples were selected only if their score value (refer to Table 2-2) for the best match organism ranged between 2.3 – 3 which mean highly probable species identification.
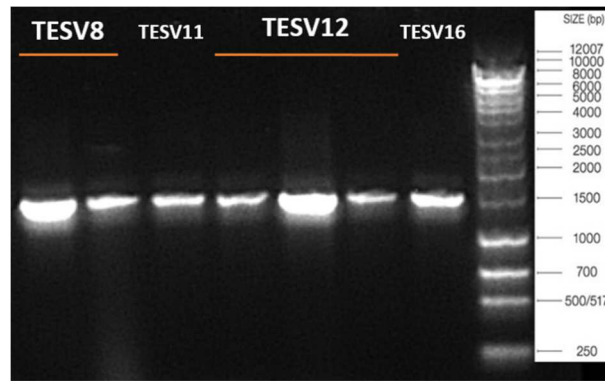
Figure 4-3: **The products of amplified PCR of *Lactobacillus* spp. from 4 different TESVs using primers 63f and 1387r** (Marchesi *et al.*, 1998).
The same primers were also used for identification of bacterial isolation. The size of the products was approximately 1,300 bp of a consensus 16S rRNA gene. PCR products underwent AGE on 1% agarose gel with Hyperladder1 plus as marker.

4.3   Interaction of TESV Community Members Through Coaggregation

Studies of coaggregation between members of the TESV biofilm community are important to establish their cellular interactions. These cell-cell interactions are often specific with adhesins and receptors involved in recognition displayed on each cell surface.

4.3.1   Identification of coaggregation between species using visual scoring

Coaggregation between species was performed according to a modified method adapted from Jakubovics *et al.* (2008a) and visual scoring was conducted using a scoring system developed by Cisar *et al.* (1979) (the detailed method is described in Section 2.4.5.1). An observation of coaggregation appears in Figure 4-4).
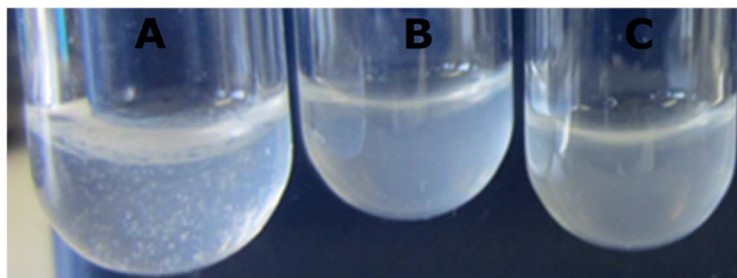
Figure 4-4: **Preliminary experiment of visual coaggregation assay.**
Samples of *S. gordonii* DLI (B, cloudy liquid) and *A. oris* MG1 (C, cloudy liquid) were used to validate the technique. When (B) was mixed with (C) and vortexed for 10 s, there was a clumping of the cells with the liquid is not cloudy. The coaggregation scoring for (A) is 4+, while for (B) and (C) scored 0, indicating that there was no auto-aggregation.

	NU34	NU19	NU25	NU27	NU31	NU30
C. albicans NU33	0	1+	2+	2+	2+	1+
C. glabrata NU34		1+	2+	2+	1+	1+
L. fermentum NU19			1+	0	2+	1+
O. anthropi NU25				0	2+	0
S. aureus NU27					1+	1+
S. oralis NU31						2+
S. epidermidis NU30						

Table 4-3: **An overview of coaggregation between clinical isolates of TESV 16.** The visual scoring was preformed using a score that rated coaggregation as 0, representing no coaggregation, to 4+ the maximum coaggregation (clear supernatant) (refer to Table 2-3).

200mM Lactose

C. albicans NU33	*C. glabrata* NU34	*L. fermentum* NU19	*O. anthropi* NU25	*S. aureus* NU27	*S. oralis* NU31	*S. epidermidis* NU30
	/	+	+	+	+	+
	NU34	+	+	+	+	+
		NU19	+	/	+	+
			NU25	/	+	/
				NU27	+	+
					NU31	-
						NU30

200mM L-arginine

C. albicans NU33	*C. glabrata* NU34	*L. fermentum* NU19	*O. anthropi* NU25	*S. aureus* NU27	*S. oralis* NU31	*S. epidermidis* NU30
NU33						
/	NU34					
-	-	NU19				
+	-	-	NU25			
-	-	/	/	NU27		
-	-	-	-	-	NU31	
+	-	-	/	+	--	NU30

Table 4-4**: The effect of sugar (lactose) and amino acid (L-arginine) on coaggregation.** Scoring system: - -, complete disaggregation; -, partial disaggregation; +, remain aggregated; /, pairs that did not coaggregate.

A screening of cell-cell interactions of isolates from TESV16 was undertaken and the data presented in Table 4-3. All bacterial species exhibited the ability to coaggregate with at least one other species. *C. albicans* NU33 and *C. glabrata* NU34 coaggregated with all bacterial species tested. *O. anthropi* NU25 scored +2 for coaggregation with both *Candida* spp. and *S. oralis* NU31 scored the same, not only with *C. albicans* NU33 but also *L. fermentum* NU19, *O. anthropi* NU25 and *S. epidermidis* NU30. However, *S. epidermidis* NU30 only formed weak cell-cell interactions when paired with either *Candida* spp., *S. aureus* or *L. fermentum*. Interestingly, the *Candida* spp. were unable to coaggregate with each other. *S. aureus* NU27 also failed to coaggregate with *L. fermentum* NU19 and *O. anthropi* NU25 but scored +2 for interaction with both *Candida* spp. *O. anthropi* NU25 was also unable to aggregate with *S. epidermidis* NU30. Any pairs that were unable to coaggregate were excluded from further testing.

Effects of the potential coaggregation inhibitors lactose or L-arginine, added into the coaggregation mix, was used to investigate the type of coaggregation interactions between species. A reversal effect provides an increased understanding of specificity of the binding as they likely consist of interactions between a protein adhesin and a carbohydrate receptor (Bos *et al.*, 1999; Kolderman *et al.*, 2015).

All 21 coaggregation pairs were tested for reversal of coaggregation by the inclusion of either lactose and L-arginine. Coaggregation was reversed in 13 pairings after incubation with L-arginine and one pair (*S. epidermidis* NU30 and *S. oralis* NU31) had a reversal effect after incubation with both (shown in Table 4-4). Interestingly, there were three coaggregated pairs (*C. albicans* NU33: *O. anthropi* NU25; *C. albicans* NU33: *S. epidermidis* NU30; *S. epidermidis* NU30: *S. aureus* NU27) that were not inhibited by either of the sugar or amino acid treatments and were considered to be insensitive to inhibition. However, this does not preclude the possibility of other sugars or amino acids inhibiting these coaggregations.

As the results in Table 4-3 show that all bacterial species were able to coaggregate with both *Candida* spp., the next step was to investigate these interactions in more detail. Heat and proteinase treatments are aimed at inactivating protein adhesins and in general, interactions that are resistant to these treatments often involve a carbohydrate receptor rather than a protein adhesin. From the observations reported

in Table 4-5, it is seen that both treatments affect coaggregations between certain bacterial species and *Candida* spp.

In almost all cases, coaggregation was reduced after treatment. Coaggregation scores in *L. fermentum* NU19 paired with heated *Candida* spp. declined, and *L. fermentum* failed to coaggregate with proteinase K (PK) treated *Candida* spp. Interestingly, the coaggregation ability of *S. epidermidis* NU30 paired with any treated *Candida* spp. remained unchanged. The same result was obtained from the association between treated *C. albicans* with *S. oralis* NU31.

TESV species	*C. albicans* NU33			*C. glabrata* NU34		
	N	85°C	PK	N	85°C	PK
L. fermentum NU19	+2	+1	0	+2	+1	0
O. anthropi NU25	+2	+2	+1	+2	0	0
S. aureus NU27	+2	+2	+1	+2	+1	0
S. oralis NU31	+1	+1	+1	+3	0	0
S. epidermidis NU30	+1	+1	+1	+1	+1	+1

Table 4-5: **Effect of proteinase and /or heat treatments on *Candida* spp. on coaggregation.**
All bacterial species were not treated (normal); N = untreated, Heat = 85°C and PK = proteinase K

The coaggregation activity of heated *C. albicans* with either *O. anthropi* NU25 or *S. aureus* NU27 was similar to interactions with untreated samples. However, both presented with finely dispersed aggregates in a turbid background when paired with PK-treated *C. albicans*. When both species were paired with heated *C. glabrata* NU34, *O. anthropi* NU25 failed to aggregate and there was a lower coaggregation score for *S. aureus* NU27. Both bacterial species were unable to coaggregate with PK-treated *C. glabrata*. *S. oralis* NU31 formed fast settling aggregates with *C. glabrata* in a slightly turbid supernatant but were unable to form aggregation with

heat or PK treated *C. glabrata*. The data indicate that in most cases, it may well be a protein on the *Candida* cell surface that interacts with either a protein or carbohydrate on the bacteria. The exception to this is the coaggregation with *S. epidermidis* NU30 which is likely driven by a *Candida* carbohydrate receptor. By contrast, *Candida* carbohydrates appear to be involved in the majority of interactions of *C. albicans* NU33 with the bacteria tested. The only exception is the coaggregation with *L. fermentum* NU19.

4.3.2 *Identification of coaggregation between species using Fluorescence microscopy imaging*

It is not possible to observe interactions between more than two species using visual coaggregation techniques. A small investigation into whether species could 'bridge' between non-coaggregating partners was done with three different species. Cells of each species were incubated with dyes and, after mixing together, were observed under a fluorescence microscope.

In Figure 4-5 there are three different dyes were used on various species. For example, a) *C. albicans* NU33 dyed green (ConA), *C. glabrata* NU34 dyed red (PI) and *S. aureus* NU27 dyed blue (DAPI) and after vortexed for 10s, coaggregation was formed as shown in the overlay. By using this technique, coaggregation between *Candida* spp. can be demonstrated with the presence of bacteria. In b) coaggregation can be observed when the species *C. glabrata* NU34 (green), *O. anthropi* NU25 (red) and *L. fermentum* NU19 (blue) formed clumps of together and seen as 'corncob' structures (Zijnge *et al.*, 2010).
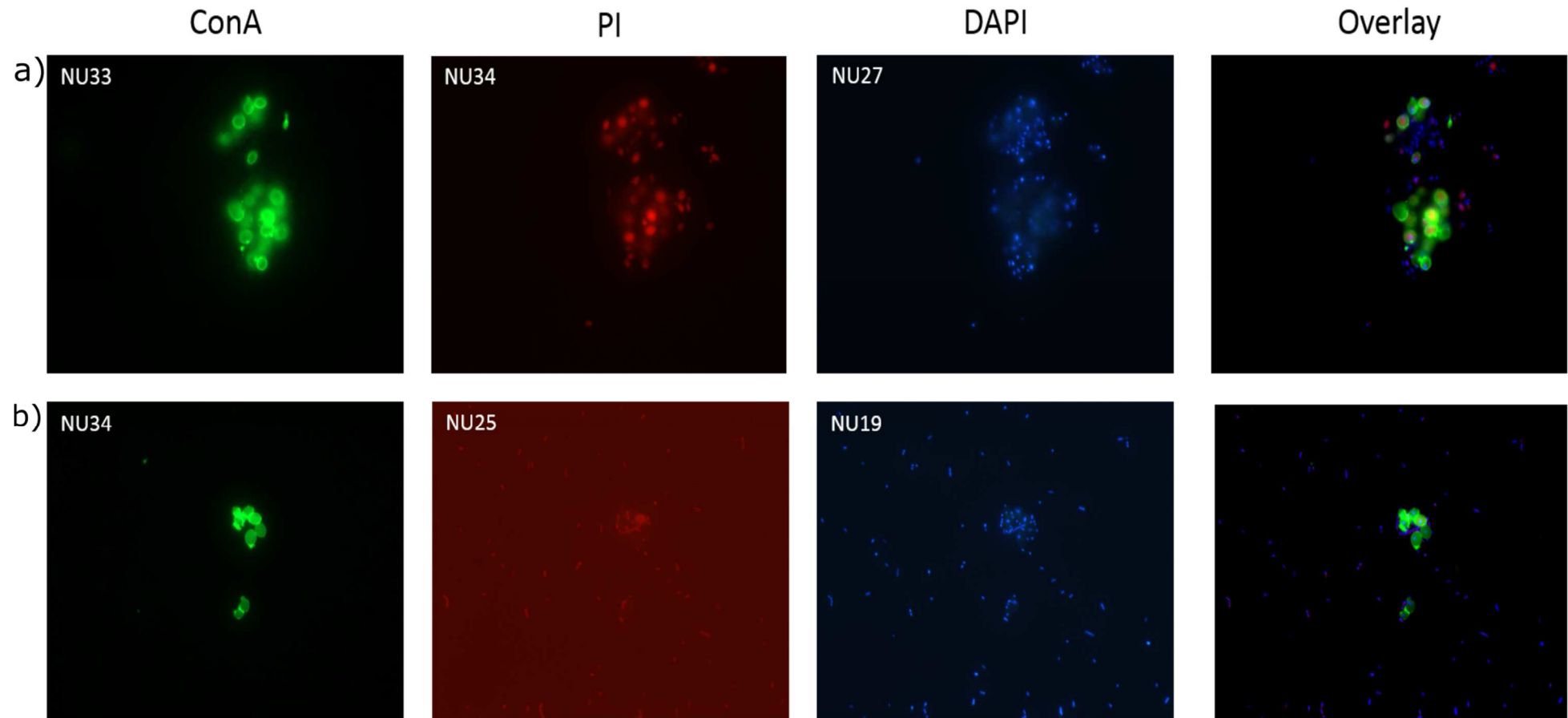
Figure 4-5: **The observation of coaggregation between TESV communities.**
Three different fluorescent dyes, PI, ConA and DAPI were used to pre-stain single species before inducing coaggregation. (a) Mixture of *C. albicans* (NU33) (green), *C. glabrata* (NU34) (red) and *S. aureus* (NU27) (blue) (b) Coaggregation between *C. glabrata* (NU34) (green), *O. anthropi* (NU25) (red) and *L. fermentum* (NU19) (blue). Magnification ×63 oil immersion

4.4 Oxidative stress

It was already established that from the TESV biofilms, several species including *Candida* spp. produced catalase, and a number of bacterial species secreted H_2O_2. Here, the potential impact of ROS production by bacteria on *C. albicans* growth and survival was investigated.

4.4.1 Production of H_2O_2 by TESV Community

The production of H_2O_2 by member species is an important factor determining biofilm composition. Production of H_2O_2 can be toxic to susceptible microbial cells and can lead to either cell death or activation of genes for survival. *L. fermentum* (TESV 8, 11 and 16), *L. paracasei* (TESV 8) and *S. oralis* (TESV 16) all produced H_2O_2 as shown in Figure 4-1.

The microbial community members of TESV 16 were evaluated for their ability to produce peroxide using a qualitative assessment of H_2O_2. The assessment in single species samples was carried out using a colorimetric assay based on conversion of an indicator, ABTS, in the presence of HRP and H_2O_2 and is shown in Figure 4-6. ABTS is a chromogen for the detection of small amounts either of peroxidase or of H_2O_2. The development of purple colour is indicative of H_2O_2 production by the colony. *S. oralis* NU31 and *L. fermentum* NU19 colonies appeared purple while the rest remained white.

Figure 4-6: *S. gordonii* **DL1 (A) and** *A. oris* **MG1 (B) grown on THB agar were incubated with ABTS, in the presence of HRP and H_2O_2.**
The purple colour on and around *S. gordonii* DL1 (A) indicates the presence of H_2O_2.

4.4.2 Effect of Environmental Stress on Candida spp.

As the isolated *Candida* spp. could successfully form biofilms alongside H_2O_2-producing bacteria, the relative stress resistant phenotypes of these *Candida* spp. compared to appropriate reference strains was examined. In addition, the resistance of these species to osmotic stress was investigated, as the production of glycerol (a response to osmotic stress) has been recently shown to be important for *C. albicans* biofilm formation (Desai *et al.*, 2013). Cell viability spot tests were performed to explore the stress resistant phenotypes of the individual *Candida* spp. Different stressors; oxidative (H_2O_2, menadione and tert-Butyl hydroperoxide (tBOOH)) and osmotic (sorbitol and sodium chloride); were used for screening.

C. albicans NU35 (TESV8), NU14 (TESV11) and NU33 (TESV16) were spotted onto YPD agar containing various stressors as shown in Figure 4-7. All *C. albicans* strains showed decreased fitness after prolonged exposure to stress conditions compared to colonies on untreated YPD plates. Although a degree of heterogeneity was observed with respect to stress resistance between the clinical isolates, most of the clinically isolated *C. albicans* strains displayed increased resistance to several oxidative stress agents compared to the appropriate reference strain. Notably, *C. albicans* NU35 showed some resistance towards the organic peroxide tBOOH, while *C. albicans* NU14 (TESV11) and NU33 (TESV16) were more resistant to both H_2O_2 and t-BOOH in comparison to the reference strain; SC5314. This is interesting because a trend towards increased resistance of the clinical isolates compared with the reference strain was not observed under other stress conditions such as superoxide or cationic stress.

A similar screening method for H_2O_2 resistance was used on clinically isolated *C. glabrata* NU15, NU34 and JG 18 (reference strain). The results, shown in Figure 4-8, indicate that all *C. glabrata* isolates were resistant to H_2O_2 even after growth in the presence of 8 mM H_2O_2 as previously reported (Cuéllar-Cruz *et al.*, 2008).

A further investigation of *C. albicans* resistance to H_2O_2 utilised a liquid assay of survival that was more quantitative and was carried out in order to measure the ability of *Candida* cell to survive short-term stress exposure. The survival graph (Figure 4-9) showed a decrease in percentage cell viability over time for all strains. However, after 90 min, 63% of *C. albicans* NU14 (TESV11) and 80% of NU33

(TESV16) cells were viable, which is considerably higher than NU35 and SC5314 cells which showed 14% and 21% survival, respectively. This observation showed that the NU14 and NU33 clinical isolates have higher resistance to H_2O_2, compared to the SC5314 reference strain, in agreement with the previous spot test result (See Figure 4-7). It is interesting that these *C. albicans* isolates can co-exist with peroxide producing bacteria in the biofilm population as shown Table 4-1.
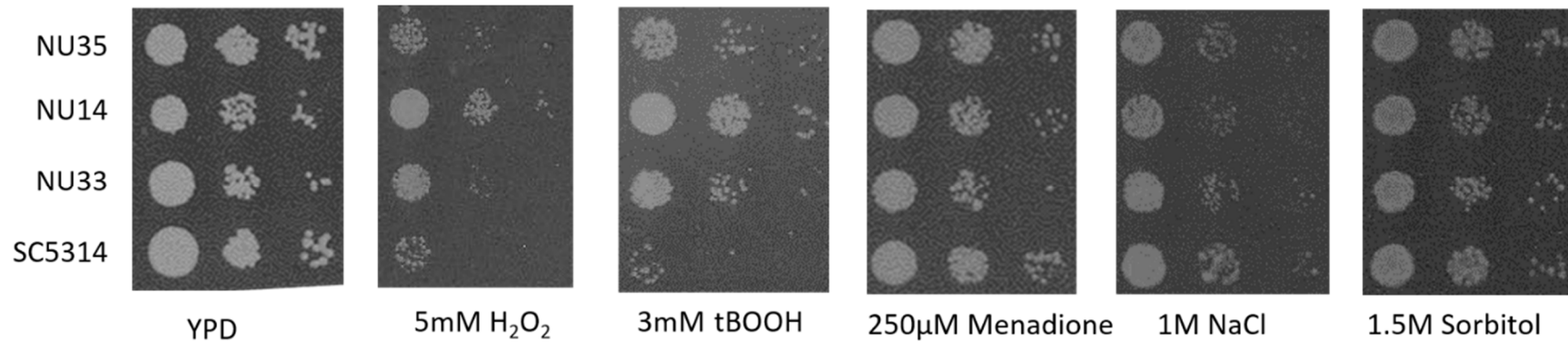
Figure 4-7: **Stress resistant phenotypes of three clinical isolates of *C. albicans* compared with the reference strain SC5314.** Exponentially growing cells were spotted in serial dilutions onto YPD agar containing the indicated stresses. Plates were incubated at 30°C for 24 h.
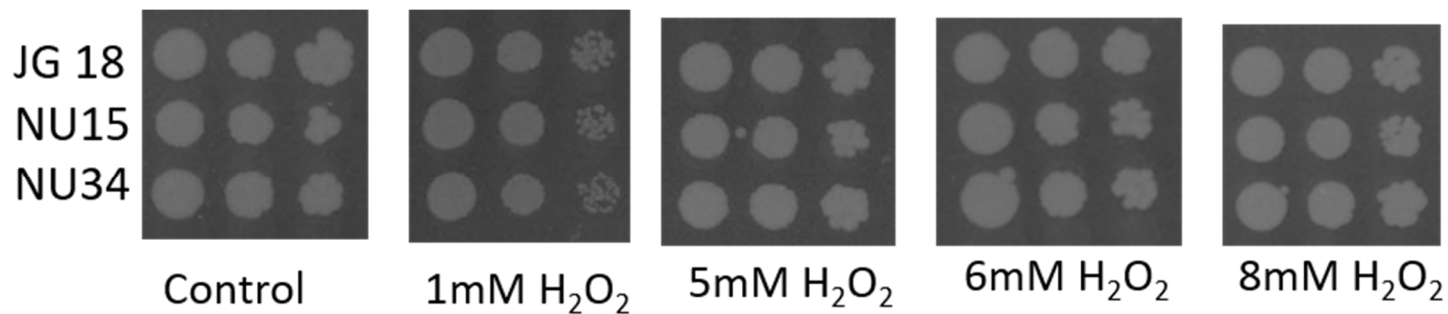
Figure 4-8: **Stress resistant phenotypes of two clinical isolates of *C. glabrata* compared with the reference strain JG18.** Exponentially growing cells were spotted in serial dilutions onto YPD agar containing the indicated stresses. Plates were incubated at 30°C for 24 h.
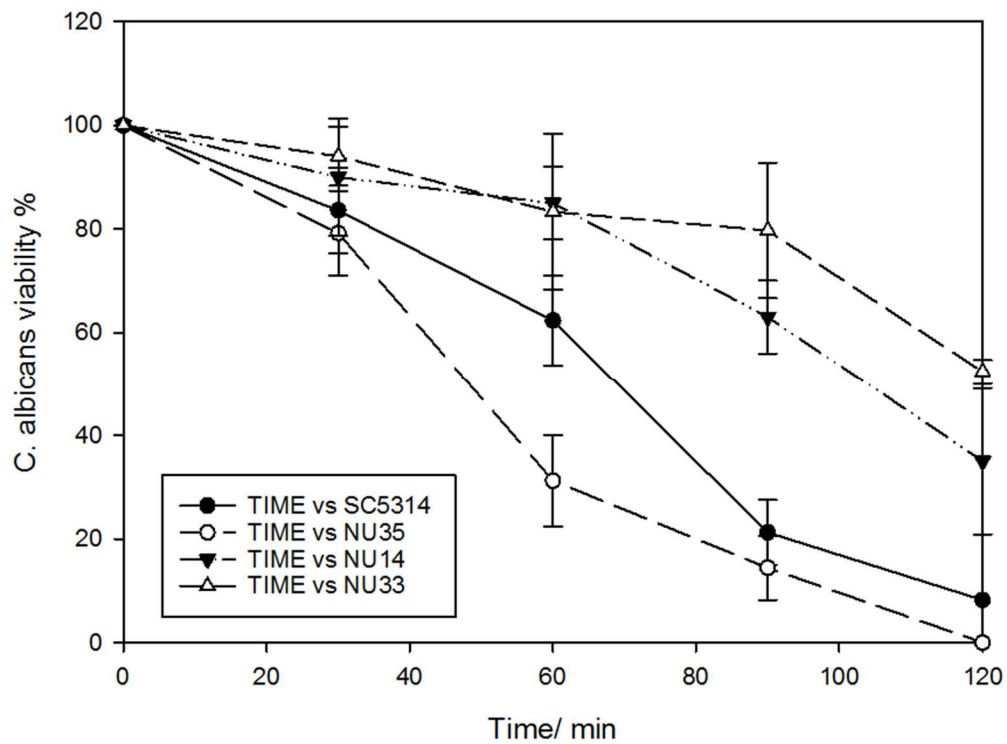
Figure 4-9: **Survival curve: *C. albicans* were grown in YPD media containing 10mM H$_2$O$_2$ and cell viability calculated over time.**
The cell viability of all strains reduced over time at different rates. NU33 and NU14 were shown to have some resistance towards H$_2$O$_2$. SC5314 is used as reference strain while NU14, NU33 and NU35 are clinical isolates from TESVs. The mean SD of two independent experiments were plotted on the graph.

4.5 Discussion

Candida albicans was shown to be present in all speech valves; either alone or in combination with *Candida tropicalis* or *Candida glabrata*. All three TESVs biofilms contained *C. albicans* and one other *Candida* sp. TESV 8 contained *C. tropicalis* and TESVs 11 and 16 *C. glabrata*. These two TESVs also shared a similar number of species isolated by culture dependent techniques and also identified in NGS. It is important to further investigate the presence of *C. glabrata* in TESV biofilms as Tati *et al.* (2016) reported that *C. glabrata* bind to *C. albicans* hyphae in the development oropharyngeal candidiasis.

Microbial community members originally from the oral cavity and tracheoesophageal areas are interchangeable and can be found on medical devices located at the area (Somogyi-Ganss *et al.*, 2017). Mitis group streptococci (i.e oral species bacteria*: S. oralis* NU31) are commonly detected in the oral cavity and the trachea in intensive care unit patients on ventilators colonized with *C. albicans* (Neu *et al.*, 1994b; Heo *et al.*, 2011; Bertl *et al.*, 2012). Mitis group streptococci and *Lactobacillus* spp produce H_2O_2 (ROS) and were commonly identified as members of TESV communities, as reported in Chapter 3. It has been reported that ROS, including H_2O_2, affect the morphology of *C. albicans* (da Silva Dantas *et al.*, 2010). Interestingly, many studies have suggested *Lactobacillus* spp. as a treatment for *Candida* related infection/ invasion and also bacterial biofilms. Biosurfactants of *Lactobacillus* sp. can act as antimicrobial and anti-adhesive agents (Zakaria Gomaa, 2013; Ceresa *et al.*, 2015). Rybalchenko *et al.* (2015) reported that *L. fermentum* exhibited an inhibitory effect on *C. albicans*, *S. epidermidis* and *S. aureus* growth. *L. fermentum* has also been suggested to suppress biofilm formation by staphylococci and *C. albicans* by cell wall and cytoplasm degradation and have the ability to disrupt mature bacterial biofilms (Rybalchenko *et al.*, 2015). The same effect was also reported on growth of *C. glabrata* (Chew *et al.*, 2015). Although these studies demonstrate an antagonistic effect of *L. fermentum* on *C. albicans* biofilm formation, our observations showed that the two can co-exist in the same community. The impact of *Lactobacillus* spp. on the TESV mixed biofilm should be further investigated in the future as it represented the majority of the bacteria in TESV (Buijssen *et al.*, 2012). While a few previous studies reported the same (or similar) groupings of species isolated from the TESV community (Shakir *et al.*, 2012; van der Mei *et al.*, 2014; Somogyi-Ganss *et al.*,

2017) (as discussed in Chapter 3), the various isolates were characterized individually or in pairs. To date no study has been conducted on combinations of all species from the community to understand the biofilm.

One of the important aspects of biofilm formation is the ability of members of communities to form physical interactions with each other, known as coaggregation. The molecular interactions that influence coaggregation often involve interactions between lectin-like adhesins and polysaccharide receptors on the partner species cell surface (Min and Rickard, 2009). Interactions may also involve non-lectin proteins that bind to proteins on the coaggregating partner cell surface (protein–protein coaggregation) (Kolenbrander *et al.*, 1995; Daep *et al.*, 2006; Sato and Nakazawa, 2014). Most studies that used induced germ tube formation of *C. albicans* when investigating coaggregation with bacteria (Jenkinson *et al.*, 1990; Bamford *et al.*, 2009; Nobbs and Jenkinson, 2015; Cavalcanti *et al.*, 2016). Arzmi *et al.* (2015) showed there is significantly reduced auto-aggregation in yeast cells compared to induced hyphae cells. For this study, SD medium was used to grow the *Candida* spp. and after an overnight culture, *C. albicans* maintained its yeast stage. *C. albicans* that were previously grown in artificial saliva were also reported to have the same finding (Arzmi *et al.*, 2015).

All bacterial species tested coaggregated with both *Candida* spp. and at least one other bacterial species as shown in diagrammatic representation (Figure 4-10). This is in agreement with the work of Cavalcanti *et al.* (2016) who showed that aggregation of *Candida* spp. can be enhanced in the presence of bacterial species within the community. O'Donnell *et al.* (2015) and Cavalcanti *et al.* (2016) found that *C. albicans* was able to aggregate with both Gram negative and Gram-positive species. *Candida* spp. anchor their position in the community by providing different receptors for binding to other bacteria. For example, *S. gordonii* interacts with *Candida* spp. via adhesin-receptors in its polysaccharide cell wall (Holmes *et al.*, 1996) and via hyphal cell wall protein Als3 (Bamford *et al.*, 2015). Host proteins such as proline-rich proteins in saliva can also assist the adherence of *C. albicans* to human surfaces e.g hydroxyapatite of tooth surface (Cannon *et al.*, 1995). However, *C. glabrata* NU34 was unable to form coaggregates with *C. albicans* NU33 although a study by Coco *et al.* (2008) showed 83% of *C. glabrata* was isolated in combination with *C. albicans* but never on its own.
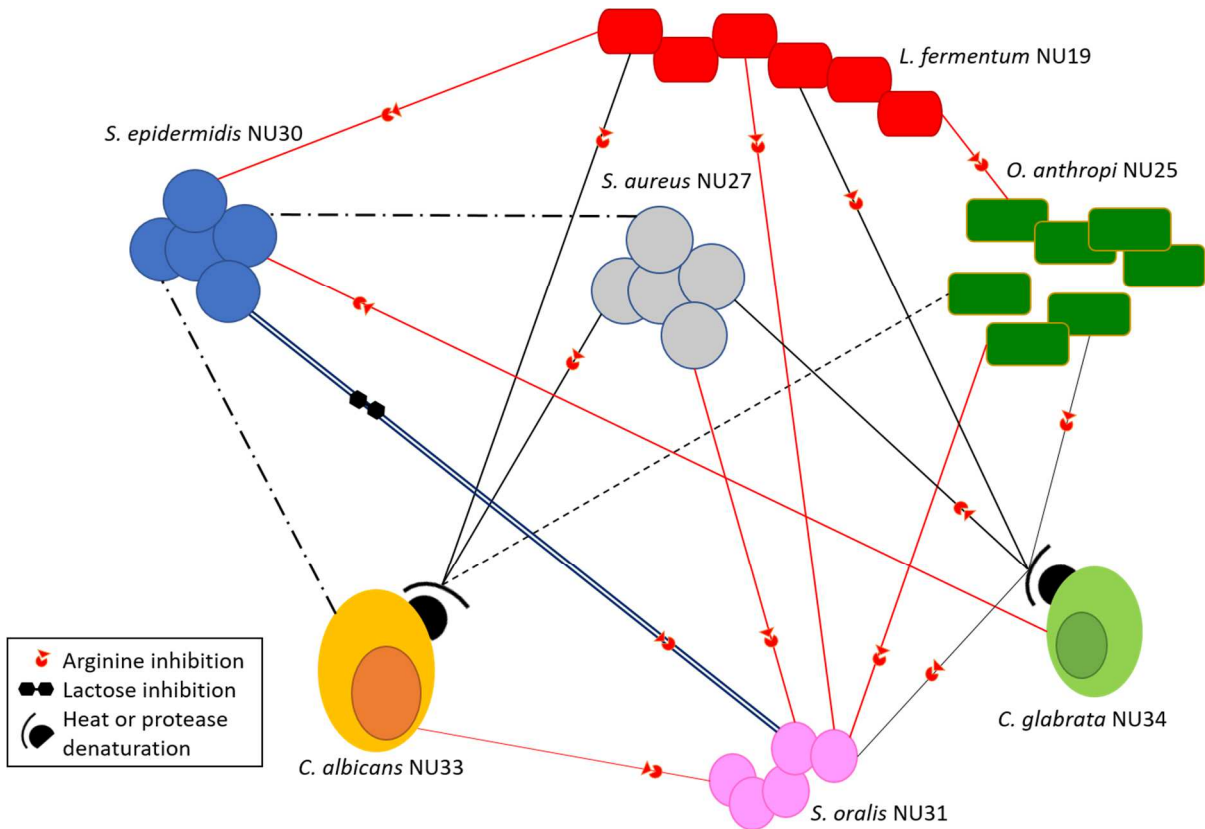
Figure 4-10: **A diagram summarising coaggregation interactions of TESV community members**.
This diagram shows coaggregations between species and indicates the inhibitions that occurred after the different treatments (incubation with L-arginine, lactose and protein denature by heat and protenaise K inhibition). All bacteria formed coaggregates with both *C. albicans* and *C. glabrata* and at least one other bacterial species. Most of the interactions were affected by L-arginine. The dotted line between *C. albicans* NU33 and *O. anthropi* NU25 indicates that the interaction was only affected by proteinase K or heat denaturation. The dotdash line between *S. epidermidis* NU30 and *C. albicans* NU33 or *S. aureus* NU27 indicates that coaggregation was not affected by treatments. While double lines with both arginine and lactose inhibition symbols indicated that both treatments affected the coaggregation between *S. oralis* NU31 and *S. epidermidis* NU30.

Although Stevens *et al.* (2015) reported that no coaggregation occurred between *S. aureus* (nasopharyngeal swab) and *S. oralis* (oral cavity) even in the presence of *C. albicans,* the pair displayed coaggregation albeit in a relatively weak association. This is in contrast with our finding that bacterial members in the community such as *S. oralis* and *S. aureus* were acting as coaggregation bridges in the TESV community, as shown in Figure 4-10. Studies also showed that many strains of *S. oralis* (Levin-Sparenberg *et al.*, 2016) and *Lactobacillus* spp. (Zakaria Gomaa, 2013) formed auto-aggregates. However, the selected clinical isolates did not auto-aggregate under the conditions employed. Another interesting finding was the ability of *S. epidermidis* to form coaggregation with all species but *O. anthropi*. Although coaggregation occurred with *C. albicans* and *S. aureus*, protein denaturation, sugar or arginine inhibition activities did not affect the interactions. Interaction of *S. epidermidis* with *C. albicans* can be suggested as lectin adhesin-receptor polysaccharide-mediated interactions because the pairs managed to coaggregate even following protein denaturation treatments of the *Candida*. Previous work indicated that the ability to form biofilms is a virulence factor of *S. epidermidis* (Costerton *et al.*, 1999). Coaggregation is often strain specific, and therefore differences between our observations and previous studies may be attributed to the strains employed (Bamford *et al.*, 2009; Arzmi *et al.*, 2015; Nobbs and Jenkinson, 2015; Stevens *et al.*, 2015; Palmer *et al.*, 2017).

The findings from this chapter set out the key physical and chemical interactions between members of selected TESV biofilm subsequently aided the development of a biofilm model to study the TESV biofilm community, as detailed in the next chapter. The mechanisms underlying the stress-resistant phenotypes identified in clinically isolated *Candida* spp are also further investigated in the next chapter.

Chapter 5. Effects of polymicrobial communities on C. albicans biofilms in vitro

5.1 Introduction

Historically, most studies on biofilms have focused on single species systems, which are relatively uncommon in the natural environment. Polymicrobial fungal-bacterial biofilms are increasingly associated with microbial infections and are commonly associated with implanted medical devices such as tracheoesophageal speech valves, heart valves, and catheters that provide artificial surfaces for biofilm growth (Douglas, 2003; Baena-Monroy *et al.*, 2005; O'Donnell *et al.*, 2015). For example, the mixed species within the oral microbiome such as *C. albicans, Streptococcus* spp*., Actinomyces* spp*. and Fusobacterium* spp. are associated with the biofilm formation on dentures and the susceptibility to denture stomatitis in denture wearers (Baena-Monroy *et al.*, 2005; Bamford *et al.*, 2009; Shirtliff *et al.*, 2009).

Researchers focusing on functional and structural aspects of commercially available indwelling and non-indwelling TESVs have employed a variety of *in vitro* models to understand the complexity of TESV biofilms. A 6-well plate static model was employed to assess the growth kinetics and colonization patterns of a *Candida* biofilm co-cultured together with *S. salivarius* on silicon disks (Leonhard *et al.*, 2013). There are also commercially available Modified Robbins Devices (MRDs) that have been used to investigate mixed species of microorganisms (Dijk *et al.*, 2000; Van Der Mei *et al.*, 2000; Schwandt *et al.*, 2004) and adapted to mimic an artificial throat with different types of TESV. (Elving *et al.*, 2003) developed a similar model, equipped with two Groningen button voice prostheses, in order to investigate mixed biofilm formation. The model was maintained at temperatures between 36.8 °C and 37.8 °C in order to mimic the environment in laryngectomized patients. In the study, clinical isolates of TESVs *C. albicans, C. tropicalis, S. aureus, S. epidermidis, S. salivarius and R. dentocariosa* were left to form biofilms for three days in the device supplied with growth medium (30% BHI and 70% yeast medium) as nutrient. Between days four and seven, the artificial throats were flushed three times a day with phosphate buffered saline and left to drain. At the end of each day, the devices were filled with growth medium for 30 min and subsequently left overnight (15 to 17 h) in the moist environment of the drained throats. The tracheal sides of the

prostheses were left in ambient air, to create cycles of feast and famine similar to the patient's situation with a stoma. Oosterhof *et al.* (2005) developed an artificial throat polymicrobial biofilm model with similar clinical isolates and measured the leakage of voice prostheses caused by the formation of biofilm. These studies demonstrated effects of biofilms on the silicone TESV itself but to date there are no detailed studies on the effect of qualitative and quantitative attributes of TESV isolated bacteria on *Candida* biofilm development in polymicrobial communities.

Changes in the environment such as high temperature, high ratio of CO_2 to O_2 , neutral pH, the presence of serum, and nutrient poor media stimulate hyphal growth (filamentation) and restrict yeast cell growth in *C. albicans* (Morales *et al.*, 2013). It has been established that formation of hyphae assisting the biofilm succession in *C. albicans* (Blankenship and Mitchell, 2006). Oral microbes like *S. gordonii* are able to promote *C. albicans* filamentation and the expression of filament- associated genes (Dutton *et al.*, 2016). RNA Seq analysis revealed that many genes involved in oxidative stress resistance were upregulated suggesting that this fungal pathogen may encounter ROS in polymicrobial communities (Dutton *et al.*, 2016). Notably, the Hog1 stress activated protein kinase (SAPK) regulates many such responses in *C. albicans* such as glycerol production, oxidative stress resistance and filamentation (reviewed in (Smith *et al.*, 2010)). However, to the best of my knowledge, there have been no reports regarding the role and regulation of *C. albicans* Hog1 or *C. glabrate* Hog1 in mixed species communities and biofilm formation.

Therefore, the aim of this section was to reconstitute a polymicrobial biofilm community, using strains isolated from a single TESV, to evaluate the effects of the bacterial presence on 1) fungal colonization in biofilm; and 2) the activation of the Hog1 SAPK pathway in *Candida* spp.

5.2 Optimization of Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The focus of this section is to establish a qPCR assay to quantify *C. albicans*, *C. glabrata* and bacteria in 7-species biofilm model. In the qPCR, an oligonucleotide probe is designed to hybridize within the target sequence of an intergenic transcribed spacer (ITS) for *Candida* spp. and universal 16s rRNA for bacteria. The cleavage of the probe by 5' nuclease activity of Taq polymerase is used to detect amplification of the target-specific product.

5.2.1 Standard plasmid preparation for qPCR

Standards for qPCR quantification of different species in mixed communities were generated using specific primers as stated in Table 2-4 to amplify fragments of *C. albicans* (NU33) and *C. glabrata* (NU34). The fragments were then cloned in pCR2.1- TOPO vector. The plasmids pCR_*Ca*1 (4038 bp) and pCR_*Cg*2 (4015 bp) were sequenced, confirming the products were appropriate targets for qPCR. The universal 16s pCR_*Pg* (5037 bp) plasmid was taken from the culture collection and has been described previously (Field *et al.*, 2012). The DNA concentrations of plasmids extracted from *E. coli* were determined by using the PicoGreen® dsDNA assay (refer to section 2.5.6).

Plasmid	Description	Size (bp)	Sources or references
pUC 19	ampr promoter, ampr, *lacZα*, pUC ori	2 686	Invitrogen
pCR®2.1	*lacZα*, f1 ori, kanr, ampr, ori	3 930	Invitrogen
pCR_*Ca*1	pCR vector containing target sequence for detection of *C. albicans*	4 038	This thesis
pCR_*Cg*2	pCR vector containing target sequence for detection of *C. glabrata*	4 015	This thesis
pCR_*Pg*	pCR vector containing target sequence for detection of *P. gingivalis*	5 037	Field *et al.* (2012).

Table 5-1: **Plasmids used during this study**.

5.2.2 DNA concentration with Quant-iT™ PicoGreen® dsDNA Assay

For absolute quantification using qPCR, it is necessary to determine the concentrations of DNA in the standards accurately. Therefore, the Quant-iT™ PicoGreen® dsDNA assay was used for quantifying plasmid DNA.

Λ DNA standards (Quant-iT™ PicoGreen kit) were diluted to create a linear standard curve (Figure 5-1). Each plasmid stock concentration was calculated from the standard curves, and concentrations were: pTOPO-*Ca*, 644 ngmL^{-1}; pTOPO-*Cg*, 478 ngmL^{-1}, and pTOPO-*Pg*, 895 ngmL^{-1}.
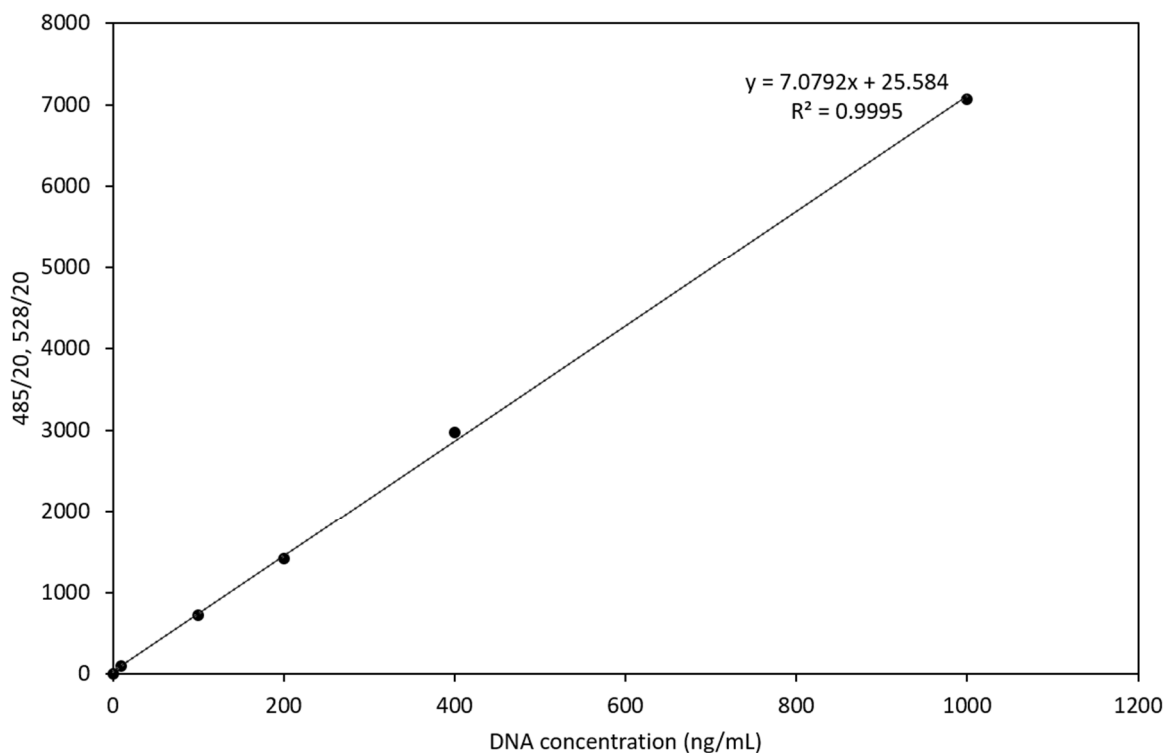
Figure 5-1**: The standard curve of PicoGreen® dsDNA assay.**
The curve was prepared by diluting λ DNA (2 µgmL^{-1}) from a range of 1 000 ngmL^{-1} to 1 ngmL^{-1} in TE buffer. Fluorescence was measured at excitation 480 ± 10 nm/ emission 520 ± 10 nm wavelengths. The total amount of DNA in ngmL^{-1} is plotted against fluorescence readings.

5.2.3 qPCR assay

The efficiency and specificity of qPCR amplification was estimated by means of standard curves shown in Figure 5-2. Selected PCR products from each run were analysed using AGE, and each product gave bands migrating at the anticipated position, indicating that PCR reactions were working as planned (refer to Figure 5-3). The reaction efficiencies (averages based on at least 3 different standard curves for each primer set) were all within the optimal range of 90 -105%.
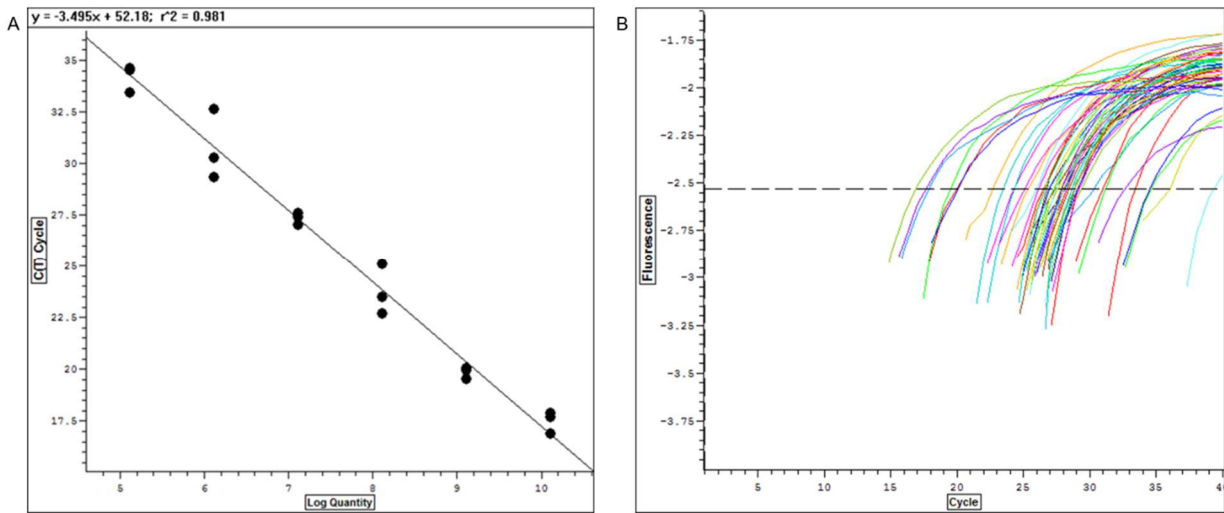
Figure 5-2: **DNA standard curves of qPCR reactions.**
Example of assay development for qPCR: analysis of *C. glabrata*. DNA standard curve (A) is the standard curve and (B) is an example of individual amplification curves that were run alongside samples during qPCR reactions as controls. The standard curve contained known ten-fold dilutions of pCR_*Cg2* (from 1.29 x 10^5 to 1.29 x 10^{10} cells mm^{-2}), which covered the range of dilutions for the samples tested. The reaction efficiencies are 93.25%.
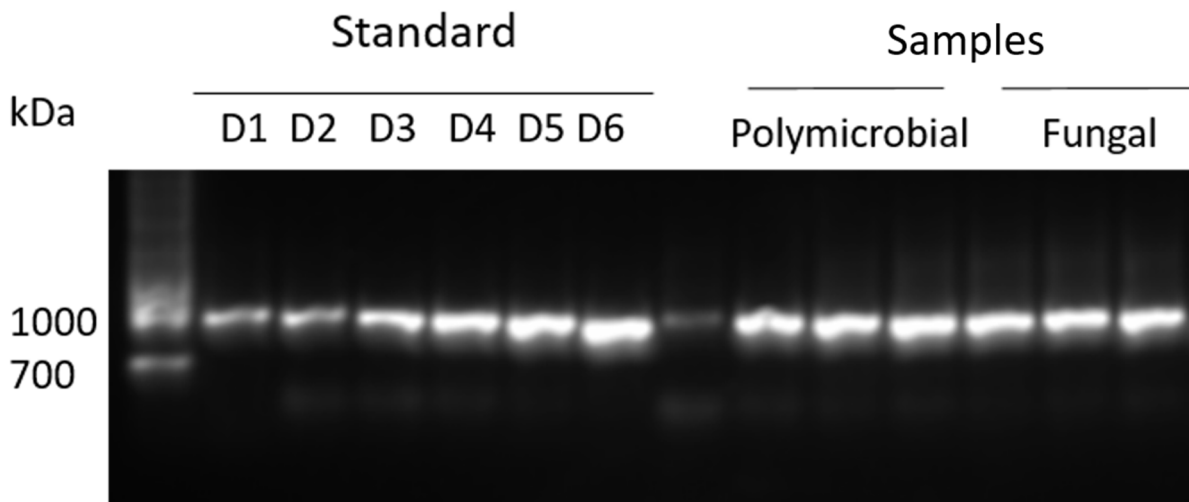
Figure 5-3: **Agarose gel electrophoresis for qPCR products of C. *glabrata* NU34.**
Ten-fold dilutions of pCR_*Cg2* standard plasmid are shown (D1= 10^2 – D6 = 10^7) and the products of *C. glabrata* NU34 qPCR (901bp). *C. glabrata* NU34 was analysed using qPCR probe and primers targeting *C. glabrata* ITS1 region. Triplicate samples of DNA extracted from *C. glabrata* NU34 in polymicrobial (with *C. albicans* NU35, *S. aureus* NU27, *S. epidermidis* NU30, *L. fermentum* NU19, *O. anthropi* NU25 and *S. oralis* NU31) and fungal (with *C. albicans* NU35) biofilm.

5.3   Optimization of Static 6-Well Plate Biofilm Model

Having developed a qPCR assay for quantifying different microorganisms, the next goal was to establish a mixed-species biofilm model (detailed method in section 2.6). Based on the microbiome data, an *in vitro* static biofilm model in the 6-well plate biofilm was developed that reflected the composition of the TESV 16 biofilm. The community members of TESV 16 included fungi represented by *C. albicans* NU33 and *C. glabrata* NU34, and the following bacteria: *S. aureus* NU27*, S. epidermidis* NU30*, L. fermentum* NU19*, O. anthropi* NU25 and *S. oralis* NU31. Fungi (10^9 cells each) and bacteria (10^8 cell each) were added simultaneously and incubated aerobically in artificial saliva at 37°C (mimicking the throat environment Elving *et al.* (2002)). Figure 5-4 shows fungal-bacterial biofilm microscopy images from an explanted TESV and from the static 6- well model. There were many similarities between the biofilm structures. In both cases, there were many different cell morphologies intermixed and syrrounded by mesh-like structures of extracellular matrix material.

Biofilms were stained with crystal violet following growth, and biofilm mass was quantified. There were no significant differences in the total amount of biofilm in mixed polymicrobial biofilms, fungal or bacterial only biofilms (See Figure 5-5). The fungal biofilm was analysed at two different times, after 24 h or 48 h. For 48 h biofilms, media were replaced after 24 h. Biofilms were harvested, and cell numbers determined by qPCR (detailed method in section 2.5.7). All the samples were snap-frozen in liquid N_2 prior to storage at -80 °C. An incubation time of 48 h was chosen for incubation of the biofilm as Figure 5-6 revealed there was a slight increase in the quantity of fungal numbers (but not statistically significant) at 48h compared with 24h incubation.
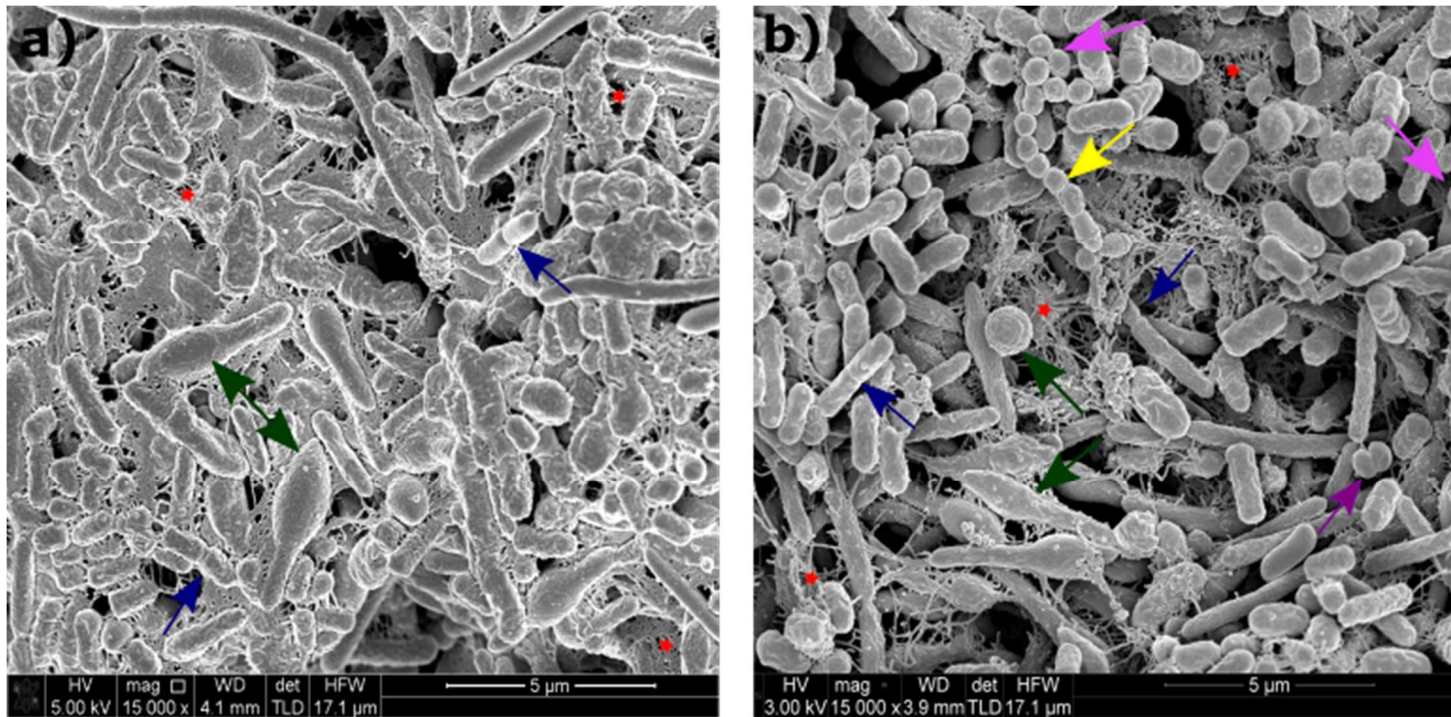
Figure 5-4: **Fungal-bacterial biofilms from a TESV and a model biofilm, imaged using FE-SEM**
Biofilms from a) an extracted TESV and b) a model fungal-bacterial biofilm. Scanning electron micrographs of each sample demonstrate a range of microorganisms such as *Candida* spp. (green arrow), long rod (blue arrow); cocci (pink arrow), diplococci (purple arrow) and chain of cocci (yellow arrow). The red stars represented mesh-like structures; biofilm matrix. a) and b) x15 000 magnification;
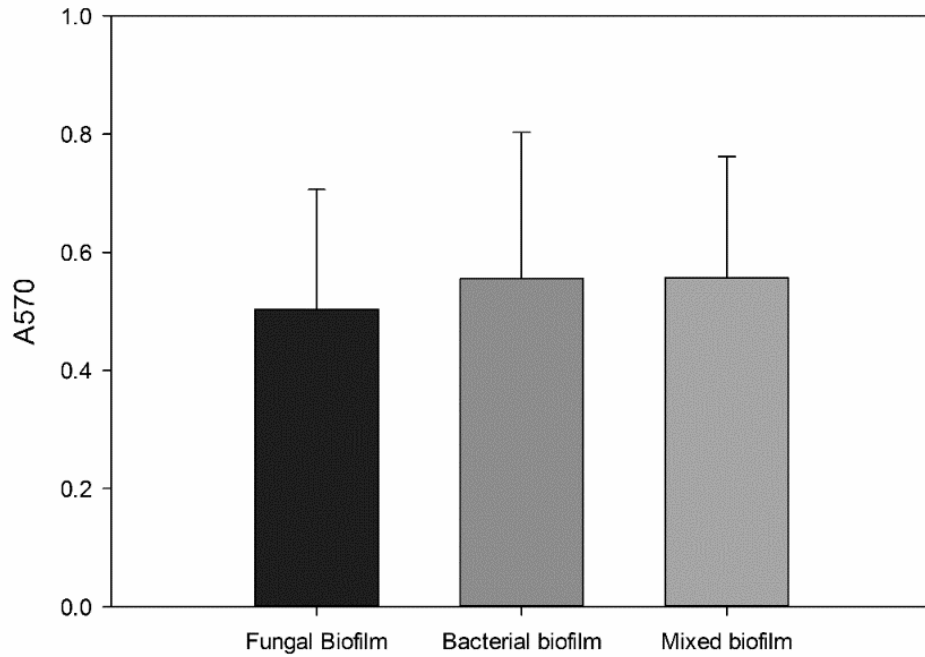
Figure 5-5: Quantification of fungal, bacterial, and mixed fungal-bacterial biofilms mass using crystal violet biofilm assay.
Bars represent mean and SDs of three independent experiments are shown. There was no significant difference between any of the samples (ANOVA p<0.05).
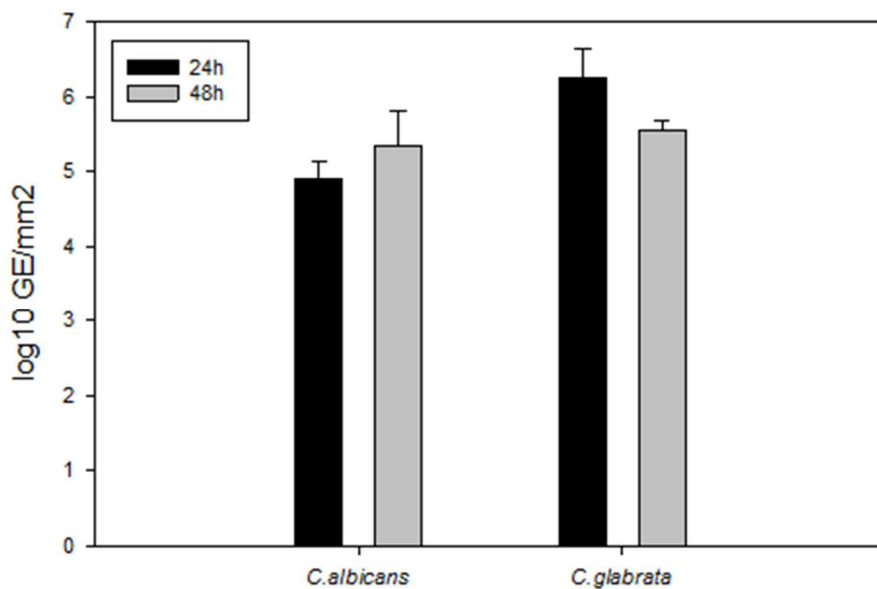
Figure 5-6: Comparison of *Candida* spp cell numbers at different incubation time using qPCR analysis.
The mean SD of three independent experiments were plotted on the graph. There was no significant difference in total cell numbers of *C. albicans* N33 and *C. glabrata* NU34 grown in fungal-only biofilm at 24 h and 48 h in artificial saliva at 37°C in 6-well static biofilm (Paired T-test; p<0.05).

5.4 Effects of Mixed Bacteria on *Candida* spp. in Biofilm

5.4.1 Fungal and polymicrobial biofilm compositions

The three strains of *C. albicans* NU35, NU14 and NU33 were grown separately in monoculture biofilms, or with *C. glabrata* NU34 in mixed-fungal biofilms. In both cases, *C. albicans* NU33 cells were present in significantly high numbers ($P < 0.05$) compared to other *C.albicans* spp. (Figure 5-8). These *C. albicans* strains were able to grow and form biofilms with other members of TESV 16, but this resulted in an approximately 50% reduction in the concentration of *C. albicans* in the biofilm compared with growth in monoculture biofilms (Figure 5-7).

It was noted that *C. albicans* cell numbers in biofilms were reduced upon co-culture with either *C. glabrata* or TESV-derived bacterial species. *C. glabrata* NU34 cell numbers remained unaffected when grown with various *C. albicans* strains in the biofilm. Although there was a slight decrease in cell numbers in the polymicrobial biofilms as shown in Figure 5-8 b), it was not statistically significant. Bacteria from TESV 16 reached similar levels in polymicrobial biofilms regardless of the strain of *C. albicans* present (Figure 5-8 c)).
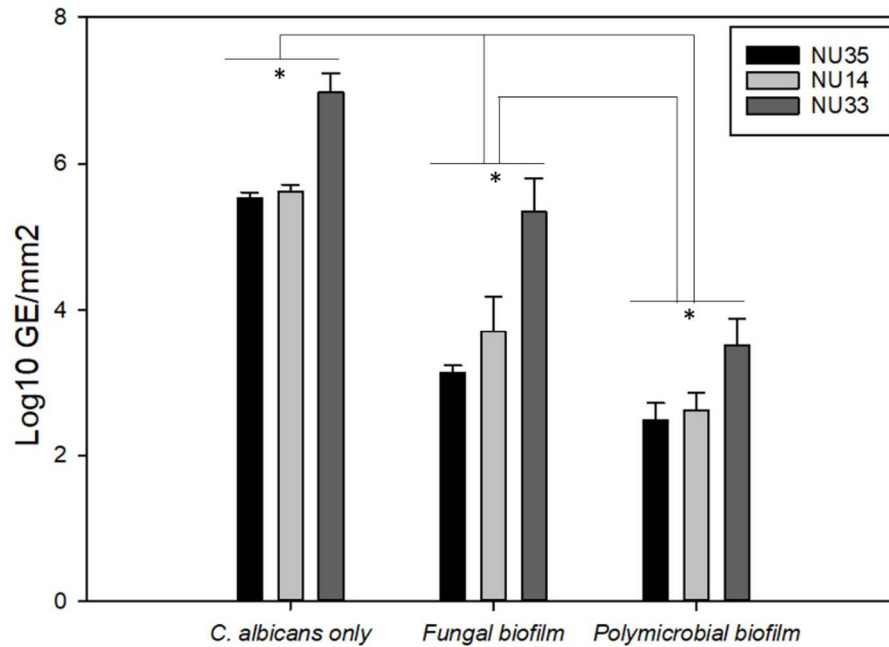
Figure 5-7: **Comparison of different *C. albicans* strains total cell number obtained from qPCR of different biofilms**.
Total cell numbers of *C. albicans* strains NU35, NU14 and NU33 in *C. albicans* only, fungal biofilm (*C. albicans* and *C. glabrata* NU34), and polymicrobial biofilms (*C. albicans*, *C. glabrata* NU34 and TESV16 bacteria). All the groups were incubated for 48h in artificial saliva at 37 $^{\circ}$C in 6-well static biofilm. The mean SD of three independent experiments were plotted on the graph. There were significantly higher numbers of *C. albicans* NU33 compared with other *C. albicans* strains (Bonferroni test for a repeated measures ANOVA, *: p<0.05).
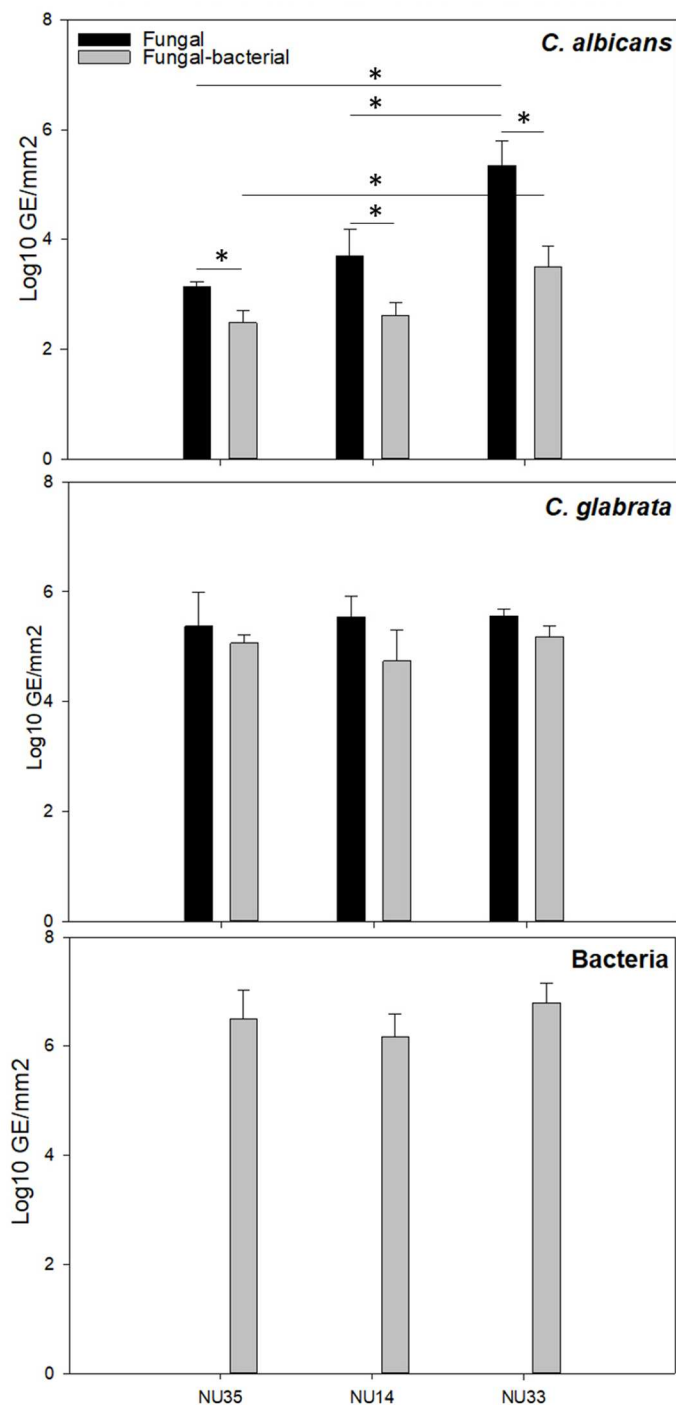
Figure 5-8: **Comparison of total cell numbers of a) *C. albicans*, b) *C. glabrata* and c) bacteria obtained from qPCR analysis of different biofilms.**
The biofilms grouped into *C. albicans* strains NU35, NU14, NU33 grown with either *C. glabrata* NU34 (fungal only biofilm) or with *C. glabrata* NU34 and TESV16 bacteria (polymicrobial biofilms). All the groups were incubated for 48h in artificial saliva at 37 °C in 6-well static biofilm. Total cell numbers of *C. albicans* and *C. glabrata* NU34 was analysed using qPCR probe and primers targeting *C. albicans* ITS2 region and *C. glabrata* ITS1 region, while total cell numbers of bacteria (*S. aureus* NU27*, S. epidermidis* NU30*, L. fermentum* NU19*, O. anthropi* NU25 and *S. oralis* NU31) were analysed using universal 16S qPCR probe and primers. The mean SD of three independent experiments were plotted on the graph. Significant differences between samples are indicated (Bonferroni test for a repeated measures ANOVA, *p<0.05).

5.4.2 Activation of the stress-activated protein kinase Hog1 in different biofilms

As described earlier, *C. albicans* must be able to adapt to the polymicrobial environment encountered within TESV biofilms, and respond appropriately to any stresses encountered. Our data indicates a reduction of *C. albicans* fitness within polymicrobial biofilms due to the observation that the number of *C. albicans* cells were reduced in the polymicrobial biofilm compared to the fungal only biofilm as shown in Figure 5-7. As described in the introductory section, the Hog1 SAPK regulates a number of responses necessary for efficient biofilm formation, therefore we examined whether this kinase was activated in both fungal only and polymicrobial biofilms. Hog1 activation can be monitored by Western blot analysis using an anti-phospho p38 antibody that recognizes the phosphorylated TGY (Thr-Gly-Tyr) motif on Hog1.

Fungal biofilms were cultivated with or without TESV bacteria as described previously (Section 2.6.2) and whole cell extracts were obtained (see Section 2.7.1). Equal amounts of protein were subject to SDS-PAGE and Hog1-P was detected by western blotting using an anti- phospho p38 antibody, after which blots were stripped and probed for total levels of Hog1 using an anti Hog1 antibody which recognises both phosphorylated and unphosphorylated forms.

The Hog1 protein of *C. albicans* can be distinguished from that of *C. glabrata* as *C. albicans* Hog1 (*Ca*Hog1) protein has a lower molecular mass (43 kDa) compared to the analogous *C. glabrata* Hog1 (*Cg*Hog1) protein (50 kDa). In all samples, the levels of *C. albicans* Hog1 were constant in both *Candida* only and mixed fungal-bacterial biofilms as shown in Figure 5-9 (protein band at 43 kDa). However, phosphorylation (activation) of Hog1 was only seen in the *Candida* only biofilm, indicating that bacteria suppress the activation of Hog1 in *Candida* biofilms. In *C. glabrata* NU34, *Cg*Hog1 could only be detected in the fungal biofilm samples, where it appeared to be phosphorylated (refer to Figure 5-9 (protein band 50 kDa)). These data indicate that bacteria suppress the expression (and possibly also phosphorylation) of Hog1.
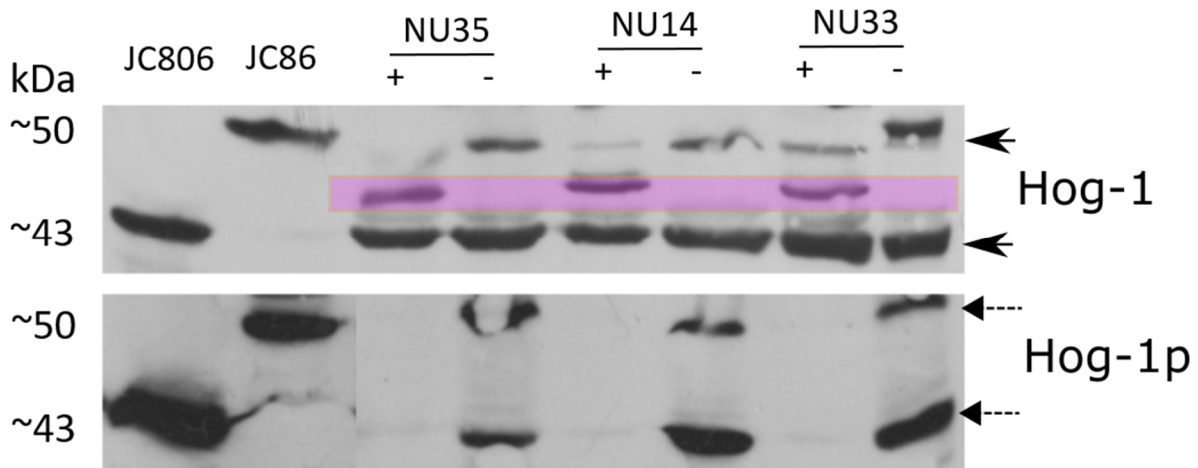
Figure 5-9: **Western blots showing phosphorylation of Hog1 in *Candida* spp.**
Western blot analysis of whole cell extracts isolated from *C. albicans* (strains NU35, NU14 AND NU33) in polymicrobial (+) or fungal (-) biofilms and *C. glabrata* NU34 grown together with different strains of *C. albicans* in fungal or polymicrobial biofilms. Blots were probed for phosphorylated Hog1 (Hog1-P) using an anti-phospho-p38 antibody and total Hog1 using an anti-Hog1 antibody. Control strains JC806 (*C. albicans hog1Δ*) and JC86 (*C. glabrata hog1Δ*) were included as negative controls. Note: solid arrows indicate Hog1 and broken arrows highlight phosphorylated Hog1 with the top arrow represented *C. glabrata*, and lower arrow for *C. albicans*. The purple bands highlight an apparent cross-reacting bacterial protein. Images show representative examples of three independent blots that all showed similar banding patterns.

Overall, therefore, the activity of the Hog1 SAPK from both *C. albicans* and *C. glabrata* was activated in fungal only biofilms. The presence of bacteria seemingly inhibits *Ca*Hog1 activation in the polymicrobial biofilm. Strikingly *Cg*Hog1 levels could not be detected in the polymicrobial film. As *C. glabrata* levels do not decrease in such biofilms this may suggest that *Cg*Hog1 protein levels are actively reduced in polymicrobial biofilms.

5.5   Discussion

In this chapter a static biofilm model was used to investigate interactions between *Candida* spp. and bacteria isolated from extracted TESVs. The use of the static biofilm is an important tool to study the early stages of biofilm formation. It is noteworthy that this system is affected by factors such as competition for adhesive sites and nutrition and thus does not allow for the formation of the mature biofilm typically associated with flow cell systems (O'Toole, 2011). This biofilm technique has been effective for identifying many factors in biofilm formation (e.g., flagella, adhesins, and metabolism) and genes that are involved in extracellular polysaccharide production (O'Toole, 2011). Published work used this method to study bacterial and fungal biofilms (Thein *et al.*, 2006) and indicated the static biofilms have some of the properties of mature biofilms such as antibiotic tolerance (Chandra *et al.*, 2001).

The total cell numbers of *C. albicans* NU33 in biofilms appeared to be significantly higher than *C. albicans* NU35 or NU14 (Figure 5-7). This can be due to the heterogenicity of *C. albicans* strains in forming biofilm as they can be HBF or LBF (Sherry *et al.*, 2014). Even though *C. albicans* NU14 numbers were higher than NU35, the numbers were not significantly different. *C. albicans* NU33 also displayed significantly higher numbers compared with *C. glabrata* NU34. It is noteworthy that *C. albicans* NU33 cells is the strain that originated from the same TESV (TESV16) as *C. glabrata* NU34, *S. aureus* NU27*, S. epidermidis* NU30*, L. fermentum* NU19*, O. anthropi* NU25 and *S. oralis* NU31. However, all *C. albicans* species, including NU33, showed a significant reduction in number when grown in mixed microbial biofilms compared with monocultures (refer to Figure 5-5 a). Consistent with this finding, van der Mei *et al.* (2014) reported that the cell numbers of *C. albicans* grown with *Staphylococcus* spp. (*S. aureus* and *S. epidermidis*), *Streptococcus* spp. (*S. salivarius*), or *Lactobacillus* spp. (*L. casei* or *L. acidophilus* or *L. crispatus*) were significantly lower than equivalent monocultures. Bacterial adhesion may reduce the subsequent adhesion of *C. albicans* and the proliferation of this fungus is affected by the diversity of biofilm community (Millsap *et al.*, 2001; Thein *et al.*, 2006).

Although different strains of *C. albicans* were co-cultured with *C. glabrata* NU34, the total number of *C. glabrata* cells was constant and this was also not affected by the

presence of bacteria in the biofilms. Tati *et al.* (2016) reported that *C. glabrata* alone was not sufficient to cause oropharyngeal candidiasis in a murine oral infection model. The presence of *C. albicans* hyphal wall Als adhesins trigger surface protein gene expression of *C. glabrata* and encourage attachment of cells. The ability for colonization by *C. glabrata* increases when co-cultured with *C. albicans* (Alves *et al.*, 2014; Tati *et al.*, 2016).

Although the total number of *C. albicans* cells decreased in the presence of bacteria, the bacterial cell numbers were unaffected by the different strains of *C. albicans* used in biofilm formation. Previous studies have reported that bacterial cells gain advantages when co-cultured with *Candida* spp. For example, *Staphylococcus* spp., such as *S. aureus,* which are frequently found together with *C. albicans* on extracted TESVs (Fusconi *et al.*, 2013) are reported to assist with the adhesion of *C. albicans* to silicone rubber (Millsap *et al.*, 2001). Once attached and forming biofilm, the extracellular matrix from *Candida* biofilms provides protection for species such as *S. aureus* from antimicrobials, and increases the chance for bacterial species to multiply (Kong *et al.*, 2016). Besides that, bacteria can also play important roles in the *Candida* spp. virulence. In communities where *C. albicans* co-exists with lactic acid bacteria (LAB) such as *L. fermentum* NU19 and *S. oralis* NU31, *C. albicans* adapts by switching between two heritable cell types, white and opaque, to undergo filamentation (Liang *et al.*, 2016). *C. albicans* hyphal formation is essential in the invasion of oral epithelia (Silva *et al.*, 2011) and penetration of silicone substrates (van der Mei *et al.*, 2014). Hyphae also provide attachment sites for *C. glabrata* (Silva *et al.*, 2011) and bacteria such as *S. gordonii* (Nobbs and Jenkinson, 2015).

Prophylactic treatment using bacteria (probiotics) is currently available for treating *Candida* spp. on TESV (Van Der Mei *et al.*, 2000) and candidiasis (Chew *et al.*, 2015). Interestingly, the production of lactic acid from LAB caused only minor inhibitory effects against *C. glabrata* biofilms (Chew *et al.*, 2015). In order to further understand the effect of bacteria on *Candida* spp., protein samples of fungal and polymicrobial biofilms were extracted and activation of the Hog1 SAPK was assessed in both *Candida* spp. Although the *C. albicans* cell numbers were one third lower in polymicrobial biofilm than in fungal biofilm, the protein analysis showed that both biofilms showed similar levels of *Ca*Hog1 expression. However, this protein was only phosphorylated, and therefore activated, in the absence of bacteria. At present

it is not clear how bacteria suppress the activation of *C. albicans* Hog1 or the expression of *C. glabrata* Hog1. There are known to be differences between the stimuli for activation of Hog1 in *C. albicans* versus *C. glabrata*. For example, glycerol production has been shown to be key in *Candida* biofilms and its production via Hog1 activation is stimulated by osmotic stress which decreases the turgor pressure of the cell wall (Desai *et al.*, 2013). In contrast, *Cg*Hog1 is activated by sorbic acid and involved in a signal transduction pathway that is activated by changes in the osmolarity of the extracellular environment (Jandric *et al.*, 2013). It is not clear whether different signalling pathways are involved in the suppression of phosphorylation of *Ca*Hog1 compared with the expression of *Cg*Hog1.

Chapter 6. General Discussion

In recent years there has been a tremendous increase in interest in polymicrobial biofilms, especially in those that form on medical devices (Kojic and Darouiche, 2004; Chandra *et al.*, 2005; Buijssen *et al.*, 2012; Ceresa *et al.*, 2015). An increasing number of patients are being treated with indwelling medical devices, and consequently the growth of biofilms on these devices is becoming a substantial public health problem. The treatment of microorganisms in biofilms is difficult as their growth characteristics and the surrounding material make them much more resistant to antimicrobial agents than sessile cells. In addition, pieces of the biofilm may detach from the device and translocate, leading to infection at other sites.

Tracheoesophageal speech valves are an example of medical devices where microbial fouling is a problem (Busscher *et al.*, 1994; Leunisse *et al.*, 2001; Hilgers *et al.*, 2003; Fusconi *et al.*, 2013; Talpaert *et al.*, 2014; Somogyi-Ganss *et al.*, 2017). It is becoming clear that biofilms on TESVs are polymicrobial, and it is important to understand how bacteria and fungi interact with each other to produce a robust biofilm. Until recently the predominant focus of health-related researchers and practitioners has been on combatting the fungi such as *C. albicans* that tends to dominate the TESV biofilm composition. However, there is now interest in those bacterial species are also present and may play key roles in biofilm formation and establishment (Palmer *et al.*, 1993; Van Der Mei *et al.*, 2000; Shakir *et al.*, 2012; Somogyi-Ganss *et al.*, 2017).

6.1   Overview of the Thesis

To further understand the microbial composition of biofilms on medical devices, improved collection and measurement techniques need to be developed in addition to more representative model biofilm systems to study various effects on the prostheses. The aims of this study were to understand the interactions between microbial species found together on a single TESV by isolating and characterising individual species from a range of TESVs, and reconstituting the biofilm of one selected TESV in an *in vitro* model system. This also included studies on the nature of the biofilm matrix in mixed-species biofilms.

Candida albicans is well-established as the predominant fungal species that impedes the function of TESVs by the formation of a robust biofilm (Tićac *et al.*, 2010; Buijssen *et al.*, 2012; Shakir *et al.*, 2012). In Chapter 3, our investigation revealed that non-*albicans Candida* species such as *C. tropicalis*, *C. glabrata* and other fungi such as *S. cerevisiae* are also common residents in the TESV biofilm community. These fungi were not alone as a group of bacterial species were also isolated from extracted TESVs. *C. glabrata* was only ever found in the presence of *C. albicans* and mixed species of bacteria. This is consistent with previous reports that *C. glabrata* is seldom identified on its own but is almost always present with *C. albicans* in the oropharyngeal area (Tati *et al.*, 2016). *C. glabrata* become invasive in the presence of *C. albicans* as Tati *et al.* (2016) found that even non-invader *C. glabrata* species were able to penetrate the oral epithelium in a murine model when *C. albicans* was present.

As one might predict when the culturomics approach was used to identify the member species in the biofilm a more complex composition was revealed than culture-based methods. Overall, 91 species were identified using NGS compared to only 16 species when using traditional culturing and MALDI-TOF MS alone. Among the most common species were *Lactobacillus* spp., *Streptococcus* spp., *Staphylococcus* spp. and *Klebsiella* spp., which have all been reported to be common colonisers of failed TESVs (Bertl *et al.*, 2013). These species were mapped into a phylogenetic tree and appear in Figure 3-11. It was very important to culture species, not only to validate the NGS analysis, but also to provide a rich resource for the rest of the work throughout the thesis. The work from Chapter 3 provided an idea of the most common species, overview or indication of the total microbial population proportion of isolated species and (most importantly) a whole range of clinical isolates that could be further characterised and employed in an *in vitro* model. This information then enabled the work undertaken in Chapter 4.

Chapter 4 set out to explore the phenotypic properties of isolated species with a view to elucidating the key factors that would underpin interactions in a polymicrobial system. As the steps of biofilm formation were discussed, there are a few factors that influence the structural consortium. Key steps in biofilm formation include adhesion to the substratum of a surface and between community members. Co-aggregation between community members will enhance the biofilm formation and lead to a

stronger structure. The presence of *C. albicans* and *C. glabrata* enhanced the co-aggregate as all bacterial species were able to attach to yeast cells of both *Candida* spp. and acted as bridging microbes between few bacterial species that were unable to co-adhere to each other. Interestingly, previous reports indicate that *C. albicans* are not only capable of attaching to bacteria but can also penetrate the surface of silicone and change the structure of TESV (Neu *et al.*, 1993; Leonhard M *et al.*, 2014). Besides *Candida* spp., bacterial members in the community such as *S. oralis* NU31 and *S. aureus* NU27 also acted as coaggregation bridges in the TESV community. As morphogenic conversions of *C. albicans* between yeast and filamentous forms of hyphal growth are important in its pathogenicity, the ability of bacteria to attach to both forms is very important. Some bacteria can attach to the hyphae of *C. albicans* (Diaz *et al.*, 2012b; Nobbs and Jenkinson, 2015) and others adhere to the yeast cell form (Arzmi *et al.*, 2015). These interactions are thought to be important in the establishment of polymicrobial biofilms.

This study aimed towards the development of a representative biofilm model of community members isolated from single explanted TESV that can be used to understand more about one particular biofilm community and to visualize the biofilm. In chapter 5, a simple static polymicrobial biofilm model was established and two species of *Candida* spp. were individually quantified along with the total bacterial population. From the observation of the polymicrobial biofilm, *C. albicans* growth was affected by the presence of bacteria. Previous studies have revealed that certain bacterial products can increase or decrease fungal growth, filamentation and biofilm formation (Jarosz *et al.*, 2009; Vílchez *et al.*, 2010; Jack *et al.*, 2015). Some bacteria benefit from the presence of *C. albicans*. For example, Kong *et al.* (2016) showed that in a mixed biofilm, there was significantly enhanced tolerance by *S. aureus* to drugs in the presence of *C. albicans* or its secreted cell wall polysaccharide material. These authors also demonstrated impairment of drug diffusion through the mixed biofilm matrix using fluorescence confocal time-lapse microscopy. The bacterial-fungal relationship goes two ways as *S. epidermidis* in a biofilm produced slime or mucoid extracellular polymers that acted as a protective agent for *C. albicans* against the action of fluconazole (anti-fungal) (Adam *et al.*, 2002). These interactions result in increased in pathogenic potential when growing in mixed biofilms as they initiate higher invasion rates, increased damage of host cells and higher expression of virulence genes (Diaz *et al.*, 2012b; Cavalcanti *et al.*, 2015).

Lactic acid bacteria are commonly identified with fungi in polymicrobial biofilms on medical devices (Buijssen *et al.*, 2012; Martinez *et al.*, 2016; O'Donnell *et al.*, 2016). Our analysis showed that *Lactobacillus* spp. and *Streptococcus* spp. were most commonly identified in TESV biofilms. Nevertheless, the presence of *Lactobacillus* spp. has been reported to give an inhibitory effect on *C. albicans*, reducing adhesion of *C. albicans* to host cells and inhibiting hyphae formation (reviewed by Förster *et al.* (2016)). These Gram-positive bacteria produced H_2O_2, caused a drop in pH and may also cause low filamentation and reduced the surface attachment by *C. albicans* and decreased the survival of *Candida* spp. (Matsubara *et al.*, 2016). These antagonistic interactions were proposed to protect the host (Wagner and Johnson, 2012). *Candida* spp. were also affected by stressors exerted by bacteria and the environment. Our findings revealed that the clinically isolated *C. albicans* were adapting and maintaining their existence in polymicrobial biofilms by becoming more resistant toward H_2O_2 and the phosphorylation of Hog1 protein was activated.

6.2 Clinical Implications and Future Work

It is already established that TESV biofilms are a mixture of fungal and bacterial species resultant from both synergistic and antagonistic reactions amongst the members. In a static biofilm model, *Candida* seem to thrive in mixed fungal biofilms containing *C. albicans* and *C. glabrata* compared to polymicrobial biofilms with both fungi and a range of bacteria. It is important to show how bacteria play a role in keeping *Candida* spp. in check. The current system for this study was static and therefore there was no removal of waste, unlike natural biofilms. There were techniques and ideas were planned for more complex and yet feasible biofilm models and quantification that can be done in the future.

In order to study TESVs community, a more accurate model biofilm, a flowing system using medical grade silicone should be developed. For future work, the study should aim to reconstitute a microbial community isolated from a single TESV to further investigate mechanisms of interaction between naturally occurring biofilm community members. One possible model would use medical grade silicone studs in a modified Robbins device (a flow cell biofilm model) with controlled temperature to imitate conditions in the throat. A rich artificial saliva media (refer to Section 2.2.1) can be employed to nourish the microbial community. A similar approach has

previously been reported (Rodrigues *et al.*, 2004; Krom *et al.*, 2009), but not using strains taken from a single explanted TESV. This is important as strains that have been isolate together have clearly been able to grow with each other in the natural TESV environment, during use by a patient. The use of our planned model, will provide a more detailed view of the interactions between species isolated from same environment, and will likely lead to more robust biofilms compared to the current static biofilm model. The microbes will not be deprived of nutrients since the medium will constantly be replenished and the flow of media might alter the composition of species. A multiplex qPCR method should be employed for quantifying bacteria and *Candida* spp. in biofilm community as this system is a simple, efficient, and cost-effective solution for limited samples and costly analysis (Gosiewski *et al.*, 2014; Jung *et al.*, 2017).

A greater understanding of species interactions may lead to new approaches for controlling TESV biofilms in future. In the speech therapy clinic, hydrogen peroxide is used to clean biofouled TESVs to salvage their function. The clinical isolated *Candida* spp. showed a resistance toward it when compared to lab strains. This observation gives a platform to investigate the mechanism of resistance, and in particular the importance of the Hog1 signalling pathway. The observation that this pathway is modulated by bacteria is very interesting. One approach would be to repeat the static biofilm model using Hog1 null strains even though this may be difficult in forming biofilm as Hog1 mutants are defective in filamentation (Alonso-Monge *et al.*, 1999)**.** There is also the need to study the impact of individual bacterial species on *Candida* Hog1 activation in biofilms. More protein analysis on *C. albicans* morphological changes should be done to understand the factors that influence this mechanism.

An extensive microscopic study using peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH) technique should be carried out to show the composition and location of different species in mixed species. In order to visualise the structure of biofilm, different PNA-FISH probes of *Candida* spp. (Machado *et al.*, 2013), universal bacteria (Shields *et al.*, 2013) and for *Lactobacillus* spp (Kempf *et al.*, 2000) have been identified and tested. This technique will provide location information, which will help to elucidate that key interactions between different species. This was attempted during the project, but the technique was unable to be

used on glass slide biofilms and images failed to develop. The biofilm detached from the surfaces during sample processing. One suggestion to overcome the detachment is by growing the biofilm on silicon stud (original idea) for a longer incubation time. The EPS matrix of the polymicrobial biofilm that included eDNA should be investigated to assess the importance of eDNA in maintaining the structural integrity of biofilms. The use of different types of DNAses such as NucB (Shields *et al.*, 2013) in treating the biofilm model can be assessed. This approach should be one focus of strategies for biofilm eradication.

6.3   Conclusion

Studies of the TESV biofilm community benefited from the microbial culturomics approach that incorporated both MALDI-TOF MS and NGS for species identification as they revealed a more complete composition than either technique alone. This is particularly important in order to develop a TESV biofilm model that accurately represents the microbial composition of natural biofilms. TESV biofilms were dominated by *Candida* spp. and harboured a mixture of bacteria and other types of eukaryote such as *Saccharomycetes*. Lactic acid producing bacteria (*Lactobacillus* spp. and *Streptococcus* spp.) normally accompanied *Candida* spp. in the biofilm. Importantly many strains in individual communities from single TESVs were able to co-aggregate in pairs, or in the presence of a bridging species. It is likely that these specific interactions are important in determining the biofilm structure *in vivo*. *Candida albicans* isolated from the TESV also showed resistance towards hydrogen peroxide compared with a laboratory strain. This observation led to further investigations on peroxide sensing and Hog1 activation. *C. albicans* appeared to be affected by the presence of bacteria in static biofilms. Further investigations will aim to build on these results to develop new strategies for controlling *Candida*-containing biofilms on TESVs

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