GENE EXPRESSION PROFILING OF THE CARBON ASSIMILATION AND VIRULENCE FACTORS OF *CANDIDA ALBICANS* IN DIFFERENT CARBON MONOSACCHARIDES SOURCES

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by

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LIST OF SYMBOLS

%	percent
C_t	cycle threshold
E	10 to the power of
g	grams
g	g-force (gravitational force equivalent)
h	hours
L	litres
m	milli
min	minutes
ºC	degrees Celcius
s	seconds
V	volts
µ	micro

LIST OF ABBREVIATIONS

ABC	Adenosine triphosphate-binding cassette
ADP	Adenosine diphosphate
ALS	Agglutinin-like sequence
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CoA	Coenzyme A
DEG	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FAD	Flavin adenine dinucleotide
$FADH_2$	Flavin adenine dinucleotide (hydroquinone form)
FDA	United States Food and Drug Administration
FDR	False Discovery Rate
GDP	Guanosine diphosphate
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
H^+	Hydrogen cation
HIV	Human immunodeficiency virus
IDSA	Infectious Diseases Society of America
IL	Interleukin
logFC	log fold change
MFC	Minimal fungicidal concentration
MFS	Major facilitator superfamily
MIC	Minimal inhibitory concentration
mRNA	Messenger ribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCBI	National Center of Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction

PDR	Pleiotropic drug resistance
P_i	Inorganic phosphate
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
SRA	Sequence Read Archive
TAE	Tris-acetate-ethylenediamine tetraacetic acid buffer
TCA	Tricarboxylic acid
$TF_{II}H$	Transcription factor II H
$TF_{III}C$	Transcription factor III complex
TNF	Tumour necrosis factor
UDP	Uridine diphosphate
USM	Universiti Sains Malaysia
YPD	Yeast Extract–Peptone–Dextrose medium
YPF	Yeast Extract–Peptone–Fructose medium
YPGal	Yeast Extract–Peptone–Galactose medium

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PROFIL EKSPRESI GEN TENTANG ASIMILASI KARBON DAN FAKTOR-FAKTOR VIRULEN *CANDIDA ALBICANS* DALAM SUMBER KARBON MONOSAKARIDA YANG BERBEZA

ABSTRAK

Salah satu faktor virulens *C. albicans* ialah kebolehlenturan metabolik yang membenarkan pertumbuhan dan penjajahan pelbagai nic di dalam perumahnya. Matlamat kajian ini adalah untuk menyiasat kesan sumber karbon berbeza dalam nic perumah terhadap pertumbuhan, kesihatan, dan kepatogenan kulat tersebut. Kesan sumber karbon yang berbeza - glukosa, fruktosa, dan galaktosa, serta kerintangan mereka terhadap ubat antikulat fluconazole, telah disiasat melalui kajian pertumbuhan, analisis ekspresi gen, dan analisis kuantitatif tindak balas berantai polimerase masa nyata (qPCR). Sel *C. albicans* menunjukkan pertumbuhan yang diperlahankan secara signifikan apabila glukosa digantikan dengan galaktosa sebagai sumber karbon mereka. Fluconazole juga didapati mempunyai kesan antikulat yang lebih besar terhadap sel-sel yang ditumbuhkan dalam glukosa dan fruktosa berbanding galaktosa. Analisis ekspresi gen menunjukkan bahawa sel-sel *C. albicans* yang ditumbuhkan dalam fruktosa dan galaktosa mengalami penyusunan semula metabolik yang besar berbanding dengan sel-sel yang ditumbuhkan dalam glukosa, menyebabkan perubahan ekspresi pelbagai gen metabolic karbon seperti *ICL1* (0.86 dalam fruktosa, -5.67 dalam galacktosa), *MLS1*(1.08 dalam fruktosa), dan *MDH1* (0.93 dalam fruktosa, -1.14 dalam galacktosa). Akhirnya, gen-gen dengan penyambungan alternatif dan transkrip baru yang diramalkan telah dapat dikenal pasti daripada sel-sel *C. albicans* yang ditumbuhkan dalam keadaan yang berbeza ini. Perubahan ekspresi gen-gen metabolik karbon menunjukkan hubungan rapat antara sumber karbon yang diasimilasikan

dengan kesihatan dan virulen *C. albicans*. Dengan pemahaman yang lebih mendalam mengenai kesan pemakanan ke atas jangkitan *Candida* dan keberkesanan rawatan antikulat terhadapnya, strategi rawatan yang lebih baik dapat dihasilkan pada masa hadapan untuk melengkapi rawatan antikulat yang sedia ada.

GENE EXPRESSION PROFILING OF THE CARBON ASSIMILATION AND VIRULENCE FACTORS OF *CANDIDA ALBICANS* IN DIFFERENT CARBON MONOSACCHARIDES SOURCES

ABSTRACT

One of the virulence factors of *Candida albicans* is its metabolic flexibility, which enables it to survive and colonise diverse niches in the host. The aim of this study is to investigate the effects of the different carbon sources available in its host niches on the growth, fitness, and pathogenicity of the fungus. The effects of different carbon sources — glucose, fructose, and galactose, as well as their resistance towards the antifungal drug fluconazole, were investigated through growth studies, gene expression profiling, and real-time quantitative PCR (qPCR). The growth of the *C. albicans* cells were significantly slowed when galactose was used as their carbon source compared to the cells grown in glucose. Fluconazole was also found to have a stronger antifungal effect towards the cells grown in glucose and fructose compared to those grown in galactose. From the gene expression profiling, it was found that *C. albicans* undergoes a massive metabolic reorganization when grown on fructose and galactose compared to when it is grown in glucose, leading to changes in the expression of various carbon metabolic genes such as *ICL1* (0.86 in fructose, -5.67 in galactose), *MLS1* (1.08 in fructose), and *MDH1* (0.93 in fructose, -1.14 in galactose). Finally, genes with alternative splicing and predicted novel transcripts were identified from the *C. albicans* cells grown in the different condition. The changes in the expressions of the carbon metabolic genes solidify a close relationship between the assimilated carbon source with the fitness and virulence of *C. albicans*. With a deeper understanding on how diet affects a *Candida* infection and the efficacy of the

antifungal treatments against it, better treatment strategies could be developed to complement current antifungal medication.

CHAPTER 1

INTRODUCTION

1.1 Problem Statement

Candida albicans is a major fungal pathogen of humans, responsible for a huge percentage of both superficial and invasive fungal infections (MA Pfaller et al., 2002). It is the most common cause of mucosal and systemic infections in humans worldwide, encompassing 70% of infection cases worldwide (Diekema et al., 2012; Guinea, 2014). Invasive *Candida* infections are the fourth most common health care–associated bloodstream infections (Wisplinghoff et al., 2004; Lewis, 2009), and are often associated with high rates of morbidity and mortality, especially since most patients with invasive fungal infections are already severely ill in the first place. The failure of antifungal treatment is a common outcome, either due to diverse fungal characteristics, tolerance or resistance towards antifungal agents, or defective host immune systems (Fisher et al., 2022).

Fungal infections have gradually become a major problem in hospitals since the 1990's, substantially increasing the global healthcare burden (G. S. Martin et al., 2003; Lepak and Andes, 2011). This threat to public health has been exacerbated by the expansion of at-risk populations and extreme global environmental changes that encourage the growth of pathogenic fungi (Fisher et al., 2022). In addition to patients with compromised immune systems and those with old age or debilitative diseases, there has been a surge in the risk of invasive fungal infections as a complication of severe COVID-19 infections (Arastehfar et al., 2020; Garg et al., 2021). Currently, there has been an increase in the number of clinical *Candida* isolates that are resistant towards first-line and second-line antifungal drugs, such as fluconazole and the echinocandins (Lockhart et al., 2012; Vallabhaneni et al., 2015). Despite the

availability of treatments, the mortality rate for *C. albicans* infections is close to 40% (Basmaciyan et al., 2019; H. Chen et al., 2020). The treatment of *C. albicans* infections is also made more challenging by the smaller array of antifungal agents available compared to antibacterial drugs, as most antifungal drugs available are mainly from just three classes — azoles, polyenes, and echinocandins (Pappas et al., 2009; Pappas et al., 2016). Once antifungal treatment failure occurs, the patient has limited options in the treatment of their infection.

The pathogenic fungus is highly adaptable and have a high chance of developing resistance following prolonged exposure to antifungals. This situation happens to a larger extent with the antifungal drug fluconazole, which is the drug of choice for antifungal prophylaxis to prevent fungal infections in immunocompromised individuals (Slavin et al., 1995; Eggimann et al., 1999). The usage of lower doses of antifungal drugs or the development of complementary antifungal strategies would ameliorate the situation with the rise of antifungal drug resistance among pathogenic fungi. Increased knowledge on the antifungal resistance mechanisms, including the changes in gene expression levels under the influence of antifungal drugs, would also allow for the nurturing of new antifungal therapeutic directions to modulate or circumvent antifungal resistance.

1.2 Significance of the Study

C. albicans must assimilate nutrients acquired from its host environment in order to grow and proliferate. Its metabolic flexibility allows it to survive the nutrient-limited niches in the host, such as in the bloodstream and within the organs (Brown et al., 2007). This metabolic flexibility also allows the fungus to survive and evade the

host's immune system during an infection (Lorenz and Fink, 2001; Prigneau et al., 2003; Lorenz et al., 2004). Therefore, *C. albicans*'s metabolic flexibility is an important virulence factor for the fungus.

While glucose is a preferred carbon source for *C. albicans*, its metabolic flexibility allows for the utilisation of other carbon sources such as fructose and galactose, along with other non-fermentable carbon sources. The assimilation of these carbon sources require the use of the yeast's gluconeogenesis and glyoxylate cycle (Lorenz and Fink, 2002). The glyoxylate cycle, especially, is required for fungal virulence, as not only is it involved in the yeast's growth and survival, it is also involved in assisting the yeast in the evasion of the host's immune system during an infection (Lorenz and Fink, 2001; Prigneau et al., 2003; Lorenz et al., 2004). Therefore, a study on the expression of the glyoxylate cycle genes that are affected when the *C. albicans* cells were grown in different monosaccharide carbon sources, and also when they were under the influence of antifungal agents, would provide a better understanding of the pathogenic yeast's growth, metabolic flexibility, and virulence.

Resistance to azole antifungal agents in *Candida albicans* is mostly conferred through mutations on genes related to ergosterol biosynthesis and cell membrane drug transporters, from the gain or loss of function of the genes, to the overexpression or underexpression of their gene products to counteract the effects of the azole antifungals. A study on the increase and decrease in the expression of these genes under the influence of fluconazole would provide a more in-depth understanding on the mechanism of action and development of azole resistance in *C. albicans*, along

with whether a change in the monosaccharide carbon source would affect the expression of these genes.

This study would also demonstrate if the pathogenicity of *C. albicans* is affected by the monosaccharide carbon source available in its environment. It is important to investigate the factors and mechanisms the yeast's metabolic flexibility and the way it affects its pathogenicity precisely because of the wide range of factors that affect it. A huge portion of the human diet consists mostly of glucose or fructose. With increasing incidences of antifungal drug resistance among clinical *Candida* isolates, complementary strategies in the treatment of *Candida* infections, such as a change in diet, could lead to a more effective treatment of the infections in synergy with the use of antifungals. The reduced usage, as well as lowered doses of antifungal drugs in combination with the other treatments of fungal infections would lead to the amelioration of the rise of antifungal drug resistance. In this study, the effects of the monosaccharide carbon sources on the yeast's resistance towards azole antifungal agents such as fluconazole were investigated.

1.3 Hypothesis

Compared to the preferred carbon source of *C. albicans*, which is glucose, the change in carbon source to either fructose or galactose would lead to a major metabolic reorganisation that would affect the growth and antifungal drug resistance of the fungus.

1.4 Objectives

1. To observe the changes in growth of *C. albicans* in fructose and galactose compared to its growth in glucose.

2. To investigate the effects of the antifungal drug fluconazole on *C. albicans* that were grown in either glucose, fructose, or galactose, via the determination of their minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC).

3. To analyse the differential expressions of *C. albicans* genes via sequencing the total RNA of *C. albicans* cells grown in either glucose, fructose, or galactose, as well as under the effects of fluconazole or not.

4. To affirm the effects of the carbon sources the *C. albicans* cells were grown in on various carbon metabolism, catabolism, and transport genes, as well as virulence and antifungal drug resistance genes.

5. To predict the *C. albicans* genes with alternative splicing and novel transcripts when grown in either glucose, fructose, or galactose, and whether or not they are under the influence of fluconazole.

CHAPTER 2

LITERATURE REVIEW

2.1 Candida albicans

Candida albicans is a unicellular, yeast-type fungus of the Saccharomycetaceae family, with a size of 4 to 6 µm. It is a dimorphic yeast, capable of existing in the oval blastospore form or in the elongated, filamentous form as pseudohyphae or true hyphae (Lo et al., 1997). *C. albicans* reproduces by budding while in the blastospore form. When grown on agar, *C. albicans* grows as white colonies with a smooth and creamy appearance. The usual growth media for *C. albicans* are yeast extract, brain heart infusion, and Sabouraud agar.

The *Candida* species are part of the human microbiota. They are considered endogenous pathogens as 40 to 60 % of healthy adults have them in the mucosal epithelium, especially in the oral cavity, gastrointestinal, and urogenital tracts (Odds, 1987; Ruhnke, 2002; Kumamoto et al., 2020). They can be found in the soil, vegetables, animals, and on the surfaces of inanimate objects.

While there are around 200 species of *Candida*, only a number of them are pathogenic towards humans, such as *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, and *C. auris* (Howell et al., 2015). The *Candida* species are the predominant pathogens associated with fungal infections in the hospital, particularly in the intensive care unit (Bille et al., 2005).

2.2 *Candida* and *C. albicans* Infections

C. albicans is an opportunistic pathogenic yeast (Gow and Yadav, 2017). Even though *C. albicans* is part of the human microbiota, *C. albicans* infections could occur with a change in the host's resistance or the host's normal flora. Most infections are

caused by an interplay between the risk factors, their interaction with other microorganisms in their niche, and the total quantity of microorganisms present (Krause et al., 1969). Infections caused by the *Candida* species are named as candidiasis or candidoses, with *C. albicans* being the most common infection pathogen of the *Candida* fungi (Garber, 2001).

Superficial candidiasis consists of common oral and genital *Candida* infections such as thrush and vaginal candidiasis. Candidiasis in the mouth and throat is called thrush or oropharyngeal candidiasis, while candidiasis in the oesophagus is oesophageal candidiasis or *Candida* esophagitis. Oesophageal candidiasis is one of the most common infections in people living with the human immunodeficiency virus (HIV) (Epstein and Polsky, 1998). The *C. albicans* found in a woman's vagina could overgrow and develop into vaginal candidiasis, an infection that affects around 3 out of 4 women at some point of their lives (Egan and Lipsky, 2000). It is also known as thrush, vulvovaginal candidiasis, or candidal vaginitis.

Invasive candidiasis, *Candida* infections that are far more severe, consists of candidemia and deep-seated tissue candidiasis such as infections in the heart, brain, eyes, and bones. Candidemia, a *Candida* bloodstream infection, is the more common form of invasive candidiasis (Kullberg and Arendrup, 2015). Candidemia is rarely found in healthy individuals, but instead is commonly found in individuals with health issues that lead to the suppression of the immune system (Yapar, 2014). It is a common infection in hospitalised patients, with approximately 25, 000 cases in the United States each year (Tsay et al., 2020).

Another form of invasive candidiasis, deep-seated tissue candidiasis, is a systemic *Candida* infection of the internal organs. Infections can be caused by the direct inoculation of the fungus to a sterile site, or by the dissemination from bloodstream

candidemia. Just like candidemia, deep-seated tissue candidiasis occurs in individuals with compromised immune systems. A majority of cases of deep-seated tissue candidiasis are linked to a co-existing or previous *Candida* infections, having evolved from candidemia. Systemic *Candida* infections are more frequently caused by *C. albicans* compared to the other *Candida* species (Epstein and Polsky, 1998).

2.3 Symptoms of *Candida* Infections

Candidiasis typically appears as white patches. *C. albicans* may appear as budding yeast cells or as pseudohyphae during infection. In oral candidiasis, white lesions are formed with painful, inflamed areas below the patches, also known as thrush. Those infected would feel burning, sensitivity, and experience altered senses of taste and smell. Infections that extend to the oropharynx will also cause difficulty and pain when swallowing. Erythematous oral candidiasis presents in the form of inflammation and the loss of papillae on the tongue (Epstein and Polsky, 1998). Meanwhile, vaginal candidiasis is associated with intense itching and a thick discharge, along with inflammation and swelling of the vagina and vulva.

Tissue-invasive infections are associated with *C. albicans* of the pseudohyphae morphology. The most common symptoms of invasive candidiasis are fever and chills that does not improve after antibiotic treatment for suspected bacterial infections. Other symptoms develop as the infection spread to other parts of the body. It is also associated with the increased length of hospital stays and high rates of morbidity and mortality (Morgan et al., 2005). However, as most patients are already severely ill before the development of invasive candidiasis, it can be difficult to pinpoint the symptoms of invasive candidiasis.

2.4 Risk Factors for *Candida* Infections

In immunocompromised individuals, such as those infected with HIV, those undergoing cancer chemotherapy, and patients undergoing organ or bone marrow transplantation, *C. albicans* can cause superficial infections such as oral or vaginal candidiasis, as well as systemic infections that could be life-threatening (Ruhnke, 2002). Those with diabetes mellitus, nutritional deficiencies, and other debilitative diseases predisposes them to *C. albicans* infections. In addition, age is also a risk factor for *C. albicans* infections, both young and old. Newborn babies often acquire *C. albicans* infections at birth, which could manifest as oral candidiasis or a diaper rash, while old age itself is a risk factor of systemic candidiasis (Weerasuriya and Snape, 2008).

In addition to the other candidiasis risk factors, the wearing of dentures, high-carbohydrate diets, nutritional deficiency, the use of cigarettes, impaired salivary gland functions, and the disruption of the oral mucosa also increase the risk for oropharyngeal candidiasis (Budtz-Jörgensen, 1990). Oral candidiasis is also associated with patients with reduced salivary flow such as those with Sjögren's syndrome, and with the use of steroid inhalers for the treatment of asthma (Fukushima et al., 2003; Radfar et al., 2003). Meanwhile, vaginal candidiasis is caused by changes in the conditions within the vagina such as pH and hormones, medicines, or changes in the immune system. Pregnancy, diabetes, the use of hormonal contraceptives or antibiotics, and having a weakened immune system are all factors that may increase the chances of vaginal candidiasis.

Candida sp. can enter the bloodstream or internal organs and cause invasive candidiasis during conditions such as surgery, the prolonged insertion of a catheter, or when the immune system has been weakened. Risk factors for invasive candidiasis include the prolonged intensive care unit stays due to critical illnesses, the use of central venous catheters, the use of broad-spectrum antibiotics, parenteral nutrition and its

length of use, having surgery or chemotherapy, having renal failure or haemodialysis, and injection drug use (Thomas-Rüddel et al., 2022). Pre-term infants with very low birth weights also have a high risk of contracting invasive candidiasis, having the combined risk factors of having vascular catheters, total parenteral nutrition, and being on antibiotics (J. Chen et al., 2016). Invasive candidiasis usually does not spread from person-to-person, but as *C. albicans* can be found on the skin, the fungus can be passed onto someone at high risk and cause an infection (Strausbaugh et al., 1994). The risk factors for candidemia are similar to those of invasive candidiasis. Causes of candidemia has also been linked to the *Candida sp.* carried on the hands of healthcare workers (Strausbaugh et al., 1994; Yildirim et al., 2007).

2.5 Virulence Factors of *C. albicans*

2.5.1 Metabolic Adaptability

The metabolic adaptability of *C. albicans* affects their susceptibility towards environmental stresses, host immune defences, and antifungal drugs (Lorenz and Fink, 2001; Lorenz et al., 2004; Barelle et al., 2006; Lok et al., 2021; Lopes and Lionakis, 2022). It enables them to survive and colonise diverse niches within the host, as many of those host niches have limited amounts of glucose and the fungus would need to turn to alternative carbon sources.

In addition, the metabolic adaptability of *C. albicans* reduces their vulnerability to environmental stresses and phagocytosis. Depending on their immediate microenvironments, individual *C. albicans* cells would undergo either glycolytic or gluconeogenic activity in renal and bloodstream infections (Fradin et al., 2003; Fradin et al., 2005; Barelle et al., 2006). They would also metabolically adapt to the nutrient-limited environment inside the host's immune cells upon phagocytosis in order to utilise

alternative carbon sources for their survival (Lorenz and Fink, 2001; Prigneau et al., 2003; Lorenz et al., 2004).

2.5.2 Biofilm Formation

The ability to adhere and form biofilms on biotic and abiotic surfaces, such as catheters, dentures, pacemakers, and prosthetic joints, is an important virulence factor of *C. albicans* (Ponde et al., 2021). The *C. albicans* biofilm matrix is composed of proteins and glycoproteins, carbohydrates, lipids, and nucleic acids. The biofilms are highly structured, consisting of multiple cell types – budding yeast cells, pseudohyphal cells, and hyphal cells (Chandra et al., 2001). *C. albicans* is the most common fungal pathogen that can form biofilms in mammalian tissues and on host-associated abiotic surfaces such as implanted medical devices.

Candida biofilms on implanted medical devices have the potential to disseminate *Candida* infections into the bloodstream, which may lead to invasive candidiasis in the bloodstream and organs. The cells dispersed from biofilms, while morphologically resembling planktonic yeast cells, are more adherent, develop a higher number of germ tubes, and are able to form biofilms more easily, all of which are factors contributing to the fungus's virulence. These cells have also been found to have increased virulence in mouse model *Candida* infections (Uppuluri et al., 2010).

In addition, *C. albicans* cells in a biofilm are also more resistant towards current antifungal drugs as the biofilm sequesters the antifungal agents and prevents them from penetrating the fungal cells (Di Bonaventura et al., 2006). *C. albicans* cells within biofilms have their drug efflux pumps upregulated regardless of the absence or presence of antifungal agents, which contributes to their increased resistance towards antifungal agents compared to planktonic yeast cells as well (Ramage et al., 2002; Mukherjee et al., 2003; Nobile and Mitchell, 2005). Furthermore, the β-1,3-glucan of the biofilm

matrix contributes to their resistance towards antifungal drugs. Exogenous β-1,3-glucans added to planktonic *C. albicans* cells increases their tolerance to the antifungal drug fluconazole, while the addition of β-1,3-glucanase increases the susceptibility of *C. albicans* biofilms to fluconazole (Nett et al., 2007; Mitchell et al., 2013). There are currently no biofilm-specific drugs, which makes the treatment of biofilm-based infections more problematic.

2.5.3 Cell Adhesion

The cell wall of *C. albicans* protects the fungus from environmental stresses such as osmotic and temperature changes, as well as from the hosts' immune system and antifungal drugs (Reyna-Beltrán et al., 2019). The cell wall is also responsible for the adhesion of the fungal cells, which is the prerequisite for the colonisation of a host niche. The adhesion stimulates hyphal formation, which in turn enhances the adhesion and invasion of the host tissues (Zhu and Filler, 2010). There is a strong correlation between adhesion and virulence, as *Candida* species with a stronger adhesion, such as *C. albicans*, are implicated in a wider range of diseases compared to *C. krusei* and *C. glabrata* with poorer adherence (H. Martin et al., 2021; Lopes and Lionakis, 2022). Biofilm formation also enables *C. albicans* to adhere to both biotic and abiotic surfaces.

Adhesins are proteins on the surfaces of the fungus that facilitate its adhesion and invasion of host surfaces. *C. albicans* cells display a huge variety of adhesins on their cell surface in order to be able to adhere to different host cell types and surfaces such as extracellular membrane and basement membranes (Tronchin et al., 2008). The adhesins also contribute towards cell surface hydrophobicity, which helps in the fungus's adherence to abiotic surfaces such as medical implants and catheters. For *C. albicans*, the Als (agglutinin-like sequence) proteins are the most widely expressed adhesins on their cell surface. The ALS genes expressed are dependent on the infection

site, as different Als proteins are better suited for adherence to different host surfaces (Cheng et al., 2005). For example, Als1 and Als3 bind to the surfaces of endothelial and epithelial cells, while Als5 binds to extracellular matrix proteins (Gaur and Klotz, 1997; Loza et al., 2004).

2.5.4 Polymorphism and Phenotype-Switching

The ability of *C. albicans* to transition between their yeast to hyphal forms is one of their virulence factors. The hyphal form of *C. albicans* is more invasive, suited for promoting tissue invasion during the early stages of infection, while the yeast form is more suited for dissemination in the blood stream during the later stages (Sudbery et al., 2004; Talapko et al., 2021; Lopes and Lionakis, 2022). Hyphal growth may be a response to nutrient deprivation, as it allows for more effective nutrient foraging (Gow, 1997). By differentiating into elongated hypha, *C. albicans* could also survive phagocytosis through the disruption of the macrophage cells (Jiménez-López and Lorenz, 2013).

There are some adhesins, such as Als3 and Hwp1, that are mainly expressed during hyphal growth (Deorukhkar and Roushani, 2017). Therefore, the phenotype switching of *C. albicans* contributes to its adhesion as a virulence factor as well. In addition, the hyphal form of *C. albicans* has the ability to release candidalysin, a hypha-specific toxin that damages the host's epithelial cell membranes for better tissue invasion and colonisation (Naglik et al., 2019). During hyphal growth, the fungus could also form asexual spores under adverse conditions to help it survive hostile environments (Ciurea et al., 2020).

In addition to its polymorphism, *C. albicans* has the ability to switch between two cellular morphologies – from round or oval white cells, to elongated opaque cells. This reversible phenotype switch is a virulence factor for the fungus as the switch to

opaque form is required for them to be mating-competent. The phenotype switch also affects other virulent factors such as the fungus's hyphae formation and susceptibility to drugs (Soll, 2014; Talapko et al., 2021).

2.6 Prevention of *Candida* Infections

The overgrowth and infection of *Candida* can be prevented by maintaining good oral and physical hygiene. Good stress management and having a well-balanced diet would also reduce the risks of *Candida* overgrowth and infections.

For those with diabetes, a good control and management of their blood sugar is important for the prevention of *Candida* infections. Avoiding unnecessary invasive devices and antibiotics would also contribute to the prevention of *Candida* infections (Budtz-Jörgensen, 1990).

Antifungal prophylaxis, which is where antifungal treatment is prescribed to a patient without the signs or symptoms of fungal disease, is typically recommended by healthcare providers to those at high risk of contracting serious *Candida* infections. In addition, as *Candida sp.* can be found on the surfaces of medical equipment and the skin, both healthcare workers and people at risk should practice infection-prevention protocols (Pappas et al., 2016).

2.7 Antifungal Treatments for *Candida* Infections

There are four main classes of antifungal drugs – polyenes, azoles, allylamines, and echinocandins. Outside of these four main classes, there are also other antifungal drugs such as ciclopirox, griseofulvin and flucytosine. The chart in Figure 2.1 displays

the types of antifungal drugs for the treatment of both superficial and invasive fungal infections.

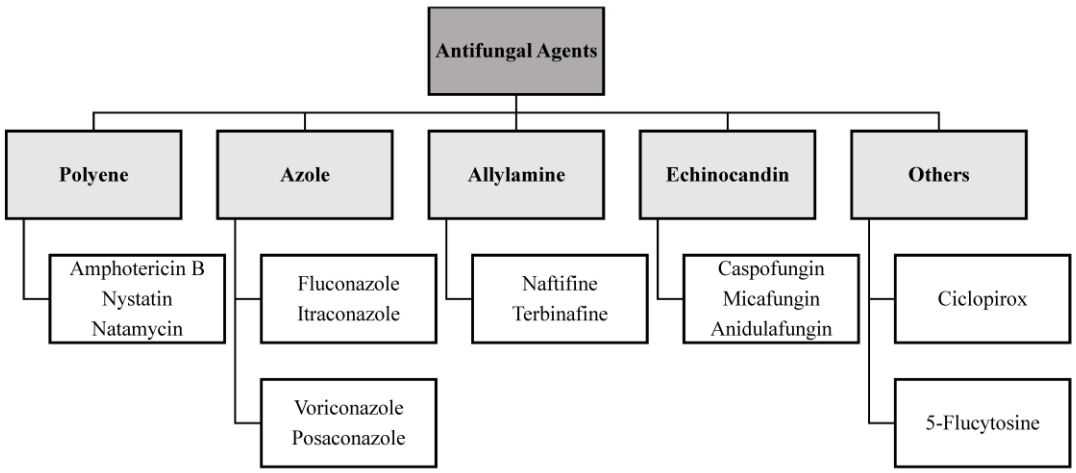

Figure 2.1 Organisational chart showcasing the types of antifungal drugs available for the treatment of superficial and invasive fungal infections

2.7.1 Polyene Antifungal Drugs

Amphotericin B, nystatin, and natamycin are polyene antifungal drugs, with amphotericin B having a long history of usage in the treatment of a wide range of systemic fungal infections. They exert their antifungal effects by binding and extracting ergosterol from the fungal cell membrane, disrupting the various cellular functions that ergosterol performs (T. M. Anderson et al., 2014). Amphotericin B is used as the last line of defence for life-threatening fungal infections in humans, as it is limited by its nephrotoxicity that causes severe and potentially lethal side effects (Walsh et al., 1998). Its usage is also limited by the requirement for intravenous administration (Laniado-Laborín and Cabrales-Vargas, 2009).

While amphotericin B is used less frequently for more resistant cases, nystatin is more commonly used for *Candida* skin infections such as oral candidiasis and vaginal yeast infections. Natamycin is used to treat fungal infections around the eye in the form of eyedrops. Nystatin and natamycin are inappropriate for oral administration as they are difficult to be absorbed via the gastrointestinal tract (Samaranayake et al., 2009).

2.7.2 Azole Antifungal Drugs

Azoles are the most widely used class of antifungal drugs. They exert their antifungal activity by inhibiting lanosterol 14α-demethylase, the fungal cytochrome P450 enzyme involved in ergosterol biosynthesis. Recent evidence suggests that they act on the cell wall structure as well (M Pfaller and Riley, 1992; Sorgo et al., 2011). The azoles can be further grouped into triazoles and imidazoles. Ketoconazole, miconazole, clotrimazole, and econazole are examples of imidazole antifungal drugs. Imidazoles are limited to treating superficial fungal infections, as triazoles have a better safety profile due to having a better affinity towards the fungal P450 enzyme compared to the mammalian counterpart (Riviere and Papich, 2013).

Fluconazole and itraconazole are first generation triazoles. They are used to treat a range of superficial and invasive fungal infections. Voriconazole and posaconazole are second-generation triazoles. Itraconazole has a wider spectrum of activity compared to fluconazole, but has significant variations in absorption and lower bioavailability (Nett and Andes, 2016). Voriconazole is structurally similar to fluconazole, while posaconazole is more closely related to itraconazole. Both has poor solubility in water, and has high variations in levels in the blood due to disparities in metabolism and absorption in the gastrointestinal tract (Girmenia, 2009). Voriconazole is active against all species of *Candida*, and is also fungicidal towards other filamentous fungi such as those of the *Aspergillus*, *Scedosporium*, *Fusarium*, and *Paecilomyces* genera. Although voriconazole is approved by the United States Food and Drug Administration (FDA) for the treatment of mucocutaneous and systemic candidiasis, it is not frequently used as it is not substantially better than fluconazole. Posaconazole has a broad spectrum of antifungal activity, and is the only azole with clinical activity against zygomycete fungi

(Torres et al., 2005). However, it does not penetrate well into the central nervous system (Girmenia, 2009).

2.7.2(a) Fluconazole

Fluconazole is an antifungal agent belonging to the triazole chemical group, in which many are fungicides and plant retardants. Fluconazole is primarily fungistatic, exerting its antifungal activity by selectively inhibiting the fungal cytochrome P-450 enzyme lanosterol 14-α-demethylase, which interrupts the conversion of lanosterol to ergosterol. Ergosterol is an essential component of the fungal cell membrane, and the disruption of ergosterol biosynthesis leads to the arrest of growth of the cells (Joseph-Horne and Hollomon, 1997).

Fluconazole is one of the most widely-used antifungal drugs for superficial *Candida* infections, such as oesophageal, oropharyngeal, and vaginal candidiasis, as well as certain systemic *Candida* infections. It is also used as a preventative medication for those at risk for developing candidiasis (Slavin et al., 1995; Eggimann et al., 1999), such as cancer patients, patients that had undergone an organ transplant, those with low blood neutrophil counts, or babies with low birth weight. It is available in both oral and intravenous formulations, and is also used for *Candida* urinary tract infections since it is highly water soluble and is excreted unchanged in the urine (Girmenia, 2009). It is well-absorbed orally, and is not affected by gastric acid or food (Thorpe et al., 1990). Compared to other antifungal agents, the use of fluconazole is more practical in the treatment of patients requiring prolonged antifungal therapy, as it is well tolerated and only needs to be taken once a day.

2.7.3 Allylamine Antifungal Drugs

Allylamines exert their antifungal activity by inhibiting squalene epoxidase, an enzyme involved in the biosynthesis of ergosterol. The inhibition leads to the

accumulation of toxic concentrations of squalene and disruption of the fungal cell membrane, resulting in an increase in cell permeability and eventually, cell death (Nowosielski et al., 2011).

Naftifine and terbinafine are classified as allylamine antifungal drugs. Naftifine only possess topical antifungal activity, while its analogue terbinafine has both topical and oral activity. Terbinafine is regularly used to treat skin and nail fungal infections. It has effective fungicidal activity against dermatophytes, moulds, and dimorphic fungi, and has fungistatic activity against *Candida sp.* (Birnbaum, 1990).

2.7.4 Echinocandin Antifungal Drugs

Caspofungin, micafungin, and anidulafungin are echinocandin antifungal drugs. Rather than target ergosterol or parts of its biosynthetic pathway, echinocandins exert their antifungal activity by inhibiting the synthesis of β-glucan, an essential component of the fungal cell wall. Echinocandins exhibit fungicidal activity against *Candida sp*., including isolates that are resistant to triazoles. They are just as effective as fluconazole or amphotericin B in the treatment of *Candida* infections in clinical settings. They have minimal drug-drug interaction, and toxicity is infrequent as their activity is specific to fungal cell walls since β-glucan is not found in mammalian cells (Morris and Villmann, 2006).

Caspofungin is available only as an intravenous formulation, and is effective in the treatment of mucosal and systemic candidiasis. Micafungin and anidulafungin are administered intravenously for the treatment of both superficial and systemic *Candida* infections. Anidulafungin also could be used in the treatment of invasive *Aspergillus* infections when used in combination with voriconazole (Grau et al., 2016).

2.7.5 Other Antifungal Drugs

Ciclopirox is a topical antifungal drug for the treatment of superficial fungal infections. While the mechanism of action of ciclopirox is not as well-understood as azoles or other antifungal drugs, it is suggested that ciclopirox may disrupt the growth and metabolism of the fungi by targeting their DNA replication, DNA repair, and cellular transport (Leem et al., 2003).

5-Flucytosine is an antimetabolite agent that is used in the management and treatment of systemic and severe *Candida* and *Cryptococcus* infections in combination with amphotericin B and/or azole antifungal drugs such as fluconazole or itraconazole. It exerts its antifungal activity by inhibiting fungal RNA and DNA synthesis, as well as disrupting the biosynthesis of certain essential proteins (Vermes et al., 2000). There are both oral and intravenous formulations available for 5-flucytosine. Unfortunately, 5-flucytosine cannot be used on its own due to the rapid development of resistance, so it is recommended to be used with amphotericin B in the treatment of systemic infections (Day et al., 2013).

2.7.6 The Usage of Antifungal Drugs for the Treatment of Fungal Infections

Topical antifungal treatments for *Candida* infections include creams, powders, mouth rinses, lozenges, and vaginal tablets. Topical preparations are the treatment of choice before systemic preparations are used as topical drugs are not absorbed systematically, thus causing less adverse side effects and lower incidences of drug-drug interactions (Epstein and Polsky, 1998).

Topical imidazole or nystatin formulations are used to treat superficial skin and mucosal fungal infections, while oral and oesophageal candidiasis are usually treated with topical clotrimazole, miconazole, or nystatin. Vulvovaginal candidiasis is treated intravaginally with azoles such as clotrimazole or miconazole. Oral or intravenous

fluconazole, itraconazole, voriconazole, or posaconazole formulations are used for more resistant or invasive infections (Maubon et al., 2014).

Currently, only three main classes of antifungals are approved for the treatment of invasive fungal infections - polyenes, triazoles, and echinocandins. For candidemia, the Infectious Diseases Society of America (IDSA) recommends an intravenous echinocandin (anidulafungin, caspofungin, micafungin) treatment for initial therapy. Alternatively, oral or intravenous fluconazole can be used for initial therapy in patients that are not critically ill and are unlikely to have infections caused by fluconazole-resistant *Candida sp.* (Pappas et al., 2009; Pappas et al., 2016). Meanwhile, caspofungin, micafungin, and anidulafungin are used intravenously for serious infections or if resistance towards fluconazole is observed (Maubon et al., 2014).

2.8 Antifungal Drug Resistance of *Candida sp.*

Unfortunately, these antifungal treatments mentioned in the previous sections are not always successful. Fungi are more closely related to humans than other pathogens, such as bacteria or viruses, which limits available antifungal targets that do not target mammalian cells as well. There has been an increase in azole-resistant *Candida* isolates in the recent years, especially towards the first-line and second-line antifungal medications – fluconazole and the echinocandins (Y. Lee et al., 2020).

The risk factors for antifungal drug resistance is similar to those of antibiotics resistance, such as immunosuppression and previous usage of antifungal agents (Garnacho-Montero et al., 2010). The rise in the emergence of the highly antifungal-resistant *C. krusei* and *C. guilliermondii* has been linked to the use of fluconazole and other azoles (Hope et al., 2002; MA Pfaller et al., 2006). Recent increases in *Candida sp.* antifungal resistance to azoles and echinocandins have led to clinical failures due to

the limited number of drug classes targeting different fungal components and the growing number of at-risk patients receiving treatment (Alexander et al., 2013; Shields et al., 2013; Maubon et al., 2014). Age is also a factor for the development of antifungal drug resistance. It was discovered that patients with fluconazole-resistant *Candida* isolates tended to be older than 15 years and had prior exposure to fluconazole. Meanwhile, patients with echinocandin-resistant isolates were younger and had prior exposure to echinocandins (Maldonado et al., 2014).

The antifungal drug resistance mechanisms include the overexpression of the protein targeted by the antifungal drug, the mutation of the target protein, the overexpression and increased biosynthesis of drug efflux pumps, and the reduction of accessibility of the target protein (Howard et al., 2020). Fortunately, the development of resistance towards polyenes are relatively rare as polyenes target a major cell membrane element to exert their antifungal action instead of an essential enzyme (Carolus et al., 2020).

2.8.1 Fluconazole Resistance of *Candida sp.*

Even though fluconazole has been a drug of choice for *Candida* infections, there has been an increase in the isolation of fluconazole-resistant *C. albicans* from patients from its widespread use, especially in HIV-infected patients who have received prior fluconazole therapy. The widespread use may have exerted a selective pressure towards the proliferation of fluconazole-resistant *Candida sp*. In addition, solid organ transplantation and neutropenia were found to be significant risk factors for the isolation of potentially fluconazole-resistant *Candida sp.* (Garnacho-Montero et al., 2010).

In addition, there has been an increase of *Candida* infections by strains with resistance or reduced susceptibility towards fluconazole such as *C. krusei* and *C.*

glabrata (Kanafani and Perfect, 2008; Perlin et al., 2015). Fluconazole resistance for *C. albicans* occurs through mutations of the *ERG11* gene, which encodes for 14-α-demethylase in steroid biosynthesis. The mutations prevent fluconazole from binding to the enzyme while still allowing for the binding of lanosterol (Asai et al., 1999; Lamb et al., 2000). The reduced affinity for the binding of fluconazole leads to decreased susceptibility towards it.

Many clinical isolates overexpress *ERG11*, which increases the target for fluconazole to bind to and thus confers a slight resistance towards it. In addition, mutations of Upc2p, a zinc cluster transcription factor that is induced upon ergosterol depletion, causes constitutive transcriptional activity and increased production of Erg11p, which also leads towards an increased resistance towards fluconazole (MacPherson et al., 2005; Heilmann et al., 2010).

Fluconazole resistance in *C. albicans* can also be tied to gene amplification due to chromosomal abnormalities. Chromosomal aneuploidy is found to be seven times more common in fluconazole-resistant isolates. *C. albicans* chromosome 5, specifically, houses the *ERG11* gene, its transcriptional regulator UPC2, and the drug efflux pump regulator TAC1 (Selmecki et al., 2006). Duplicate copies of these genes confer increased resistance towards fluconazole.

Resistance against fluconazole could also be conferred to *C. albicans* via loss-of-function mutations in *ERG3*, a gene which encodes for the ergosterol biosynthetic enzyme C-5 sterol desaturase (Martel et al., 2010). This type of mutations allows the fungus to bypass the accumulation of toxic methylated sterols produced due to the blockage of lanosterol 14-α-demethylase, which confers resistance against azole-type antifungal drugs (Cowen and Steinbach, 2008).

Fluconazole resistance in *C. albicans* isolates is often acquired step-wise over time, involving multiple of the mechanisms mentioned above. This leads to a high level of resistance observed clinically in patients undergoing long-term fluconazole treatments (Berkow and Lockhart, 2017).

2.8.1(a) The Roles of Cell Membrane Drug Transporters in the Fluconazole Resistance of *C. albicans*

The efflux of the antifungal agents out of the cell confers resistance to *C. albicans* towards the drug. Some fluconazole-resistant *C. albicans* isolates overexpress drug transporters such as CDR1 and CDR2 of the ATP-binding cassette (ABC) superfamily, and MDR1 of the major facilitator superfamily (MFS) of multidrug transporter proteins, to counter the effects of fluconazole (Wirsching et al., 2000; Siikala et al., 2010).

CDR1 and CDR2 are induced in young biofilms immediately after adherence, leading to the enhanced tolerance to fluconazole associated with increased metabolic activity during biofilm formation (Mateus et al., 2004). The overexpression of CDR1 and CDR2 is a result from the mutation of the transcriptional activator TAC1 (Tsao et al., 2009). The inactivation of TAC1 contributes to the loss of upregulation of CDR1 and CDR2, and a TAC1 allele recovered from an azole-resistant strain could trigger their upregulation in an azole-susceptible laboratory strain (Coste et al., 2004). NDT80 is another positive transcription activator of CDR1. The deletion of NDT80 increases *C. albicans* sensitivity towards antifungal drugs, and removes the drug-induced expression of CDR1 (C.-G. Chen et al., 2004). In addition to the increased transcriptional activation, increased mRNA and protein stability also contributes to the overexpression of CDR1 in the azole-resistant strains (Manoharlal et al., 2008).

Compared to CDR1, MDR1 is more specific for fluconazole efflux transport (Fling et al., 1991). Fluconazole-resistant clinical strains of *C. albicans* are frequently observed with having MDR1 activation. In addition, increased fluconazole susceptibility and decreased virulence was observed in the fluconazole-resistant isolates with disrupted *MDR1* genes (Wirsching et al., 2000). CDR1, CDR2, and MDR1 promoters are more highly expressed in adherent cells compared to planktonic cells, which confer fluconazole resistance to the *C. albicans* cells (Mateus et al., 2004).

FLU1 is a gene that encodes another multidrug efflux pump of the MFS plasma membrane transporters. It is specifically a fluconazole resistance gene, and its expression can be induced by fluconazole and ciclopirox olamine, an antifungal treatment for skin infections (Niewerth et al., 2003). Its expression does not vary between azole-susceptible and -resistant clinical isolates, which indicates that it is not involved in the acquirement of azole resistance in *C. albicans* (Park and Perlin, 2005). In addition, it is fungal-specific with no human or murine homologues, which makes it an attractive target to counter the increasing *C. albicans* resistance towards azole-type drugs.

2.9 Carbon Assimilation of *C. albicans*

2.9.1 Glucose, Fructose, and Galactose

Glucose, fructose, and galactose are hexoses, simple sugars with six carbon atoms. On the whole, glucose is the most abundant monosaccharide, and is the most important source of energy in all organisms. Glucose circulates in the blood of animals as blood sugar, which *C. albicans* obtain from their hosts. *C. albicans* have a sensitive response to glucose, even at 0.01 % (Rodaki et al., 2009). Other than being an energy source, glucose also affects their resistance to osmotic, oxidative, and cationic stresses,