Mechanisms of echinocandin resistance in *Candida* species

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Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Preface

The work presented in this thesis was performed at the Department of Microbiology of Gdansk University of Technology (Gdansk, Poland), during the period February-June 2019, under the supervision of Prof. Dr. Anna Brillowska-Dąbrowska, and within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Dr. Miguel Teixeira.

Acknowledgements

The process of writing this thesis has been one of the biggest challenges of my life. It was a product of five years of hard work and sacrifice, involving people without whom I would not have made it.

Firstly, I would like to thank my family. My parents, my brother, my grandparents and my aunts and uncles. The financial help was significant and crucial but the unconditional emotional support and love for me during these crazy years was what always kept me going and that brought me to this point. Everything I am and everything I will be is, in its greatest part, thanks to them.

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O que tem que ser, tem muita força.

Morgana Vieira de Oliveira e Castro

Contents

List of figures

List of tables

Acronyms

µL – Microliters

AFST – Antifungal susceptibility testing

AMP – Ampicillin

AND – Anidulafungin

C. albicans – Candida albicans

C. glabrata – Candida glabrata

C. inconspicua – Candida inconspicua

C. krusei – Candida krusei

C. palmioleophila – Candida palmioleophila

C. parapsilosis – Candida parapsilosis

C. tropicalis – Candida tropicalis

$CaCl_2$ – Calcium chloride

Candida spp. – Candida species

cDNA – Complementary Desoxirribonucleic Acid

CLSI – Clinical and Laboratory Standards Institute

CSP – Caspofungin

C_T - Threshold cycle

DMSO – Dimethyl Sulfoxide

dNTP – Deoxynucleotide Triphosphates

E. coli – Escherichia coli

ESCMID – European Society for Clinical Microbiology and Infectious Diseases

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FLPs – Flavodoxin-Like Proteins

h – Hours

HIV – Human Immunodeficiency Virus

IDSA – Infectious Diseases Society of America

IPTG – Isopropyl-β-D-thiogalactoside

KCl – Potassium chloride

LA - Lysogeny broth with agar

LB - Lysogeny broth

MCF – Micafungin

MDR – Multidrug Resistance

$MgCl_2$ – Magnesium chloride

MIC – Minimum Inhibitory Concentration

min – Minutes

mL – Millilitres

mM – Millimoles

$NaHCO_3$ – Sodium bicarbonate

OD – optical density

OD_{600} – Optical Densitometry at 600 nm

PCR – Polymerase Chain Reaction

RNase – Ribonuclease

Rpm – Revolution Per Minute

RT-qPCR – Quantitative Real Time Reverse Transcription Polymerase Chain Reaction

S. cerevisiae – *Saccharomyces cerevisiae*

TET – Tetracycline

UV – Ultraviolet

V – Volts

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

Echinocandins are antifungals used against *Candida* infections and resistant strains have been emerging. Among these strains are *Candida albicans* and non-*albicans*. Given the widespread use of echinocandins, it is important to study how resistance mechanisms act and evolve. Antifungal susceptibility tests for echinocandins were performed for 29 isolates of *Candida* species. Two resistant isolates were obtained, *Candida krusei* (4 mg/L MIC for the three echinocandins) and *Candida glabrata* (0.5 mg/L MIC for anidulafungin and caspofungin and 1 mg/L for micafungin) and two isolates of *Candida inconspicua* and *Candida palmioleophila* with high MIC values of 4 mg/L for the three echinocandins, something that had not been previously reported. Resistance to echinocandins is based on the occurrence of point mutations of the *FKS* gene. PCR identification using a single primer for the *FKS* gene was performed to obtain multilocal patterns that would allow quick identification. A total of 70 isolates of *Candida* species were tested. It was possible to obtain identifiable patterns for *Candida albicans*, *Candida palmioleophila* and *Candida krusei*. The last two presented different patterns according to their susceptibility to echinocandins. The genes *CHS1*, *PST1*, *CWP1*, and *CWP2* encoding cell wall proteins, possibly upregulated when there are alterations in the *FKS1* gene, were investigated for 8 *Candida* isolates. Upregulation of all genes was verified for one susceptible isolate of *Candida albicans* and two resistant isolates of *Candida palmioleophila* and *Candida krusei*, pointing to a possible alteration in the *FKS1* gene of these isolates. In general results have shown that amongst *Candida* species, there are important connections between cell wall dynamics and resistance to antifungal drugs. It is important to continue to try to understand how the mechanisms of resistance and the cellular responses to maintain cell wall integrity work in order to develop new strategies to fight these infections.

Keywords: *Candida albicans*, non-*albicans Candida* species, antifungal drug resistance, echinocandins, PCR fingerprinting, real time PCR, *FKS* gene

Resumo

Equinocandinas são antifúngicos usados contra infeções provocadas pelo fungo *Candida,* no entanto, cada vez mais, têm sido identificados isolados resistentes pertencentes a espécies de *Candida albicans* e não-*albicans*. Dado o uso generalizado de equinocandinas, é importante verificar como agem e evoluem os mecanismos de resistência. Testes de suscetibilidade antifúngica às equinocandinas foram realizados para 29 isolados da espécie *Candida*. Foram obtidos dois isolados resistentes, *Candida krusei* (CIM de 4 mg/L para as três equinocandinas) e *Candida glabrata* (CIM de 0,5 mg/L para anidulafungina e caspofungina e 1 mg/L para micafungina) e dois isolados de *Candida inconspicua* e *Candida palmioleophila* com valores elevados de CIM de 4 mg/L para as três equinocandinas, algo que não havia sido previamente observado. A resistência às equinocandinas baseia-se na ocorrência de mutações pontuais do gene *FKS*. Foi realizada a identificação por PCR usando um único primer para o gene *FKS* para obter padrões que permitissem a identificação rápida das espécies testadas. Um total de 70 isolados de *Candida* foram testados. Foi possível obter padrões identificáveis para *Candida albicans*, *Candida palmioleophila* e *Candida krusei*. Os dois últimos apresentaram padrões diferentes de acordo com a suscetibilidade às equinocandinas. Os genes que codificam para proteínas da parede celular *CHS1*, *PST1*, *CWP1* e *CWP2,* possivelmente regulados positivamente quando existem alterações no gene *FKS1*, foram investigados para 8 isolados de *Candida*. A regulação positiva de todos os genes foi verificada para um isolado suscetível de *Candida albicans* e dois isolados resistentes de *Candida palmioleophila* e *Candida krusei*, apontando para uma possível alteração no gene *FKS1* destes isolados. Em geral, os resultados mostraram que, entre as espécies de *Candida*, existem importantes conexões entre a dinâmica da parede celular e a resistência a medicamentos antifúngicos. É importante continuar a tentar entender como funcionam os mecanismos de resistência e as respostas celulares para manter a integridade da parede celular, com o objetivo de desenvolver novas estratégias para combater estas infeções.

Palavras-chave: *Candida albicans*, espécies não-*albicans* de *Candida*, resistência a drogas antifúngicas, equinocandinas, identificação por PCR, PCR em tempo real, gene *FKS*

1. Introduction

1.1. Thesis Outline

This dissertation is organized in four chapters.

The first chapter offers an overview on the increasing frequency of non-*albicans Candida* species, followed by some insight into the currently used families of antifungal drugs in clinical practice, together with their modes of action. Special attention is given to echinocandins, one of the main families of drugs presently used, with information concerning modes of action and its resistance mechanisms, point mutation in the *FKS* gene. Insight about how these mutations affect the cell wall and cell wall gene expression is also discussed.

The second chapter contains all the materials and methods used during the fulfilment of this work.

The third chapter describes the results attained with this study, first for antifungal susceptibility testing to identify *Candida* strains (*albicans* and non-*albicans*) that are resistant or susceptible to echinocandins. It further comprises the analysis of PCR fingerprinting for *Candida albicans*, *Candida glabrata*, *Candida palmioleophila*, *Candida parapsilosis* and *Candida inconspicua* and discusses the identified patterns in terms of their connection to each species and susceptibility phenotypes. Finally, it analyses the expression level of cell wall genes in three species of *Candida* and discusses the possible connection between the expression levels and alteration of the *FKS* gene.

In the fourth chapter, the results obtained with this project are discussed and compared with the current knowledge about echinocandin resistant mechanisms.

In the fifth and last chapter, final remarks considering the work developed and future perspectives are made, together with references to what contributions this work offered in the comprehension of acquired resistance mechanisms to echinocandins in *Candida* species.

1.2. Emergence of *Candida* species

Candida is a genus of yeasts and one of the most common cause of fungal infections worldwide. These microorganisms are small (3–5 µm) (1) and they reproduce by budding (2). These yeasts can be seen microscopically in the form of yeasts, pseudo-hyphae (budding cells that do not separate), or true hyphae (multicellular organisms) (Figure 1) (3). *Candida* spp. belong to the class *Ascomycetes*, order *Saccharomycetales*, and family *Saccharomycetes*.

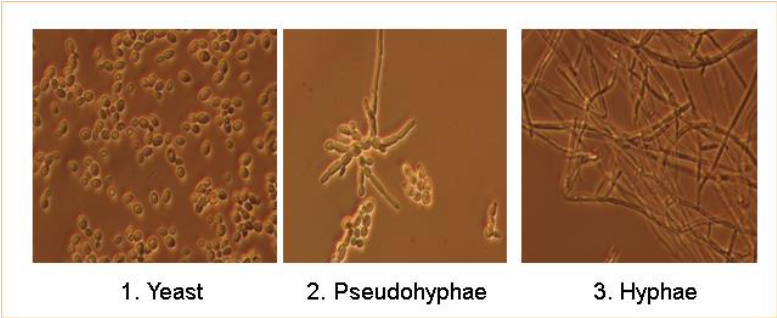


Figure 1 – Adapted from British Society of Immunology. The three morphological forms of *Candida albicans*. Yeast (1) are small, round cells that divide by conventional cell division. True hyphae (3) are elongated cells that do not separate following cell division and are separated by specialised septa that allow passage of cytoplasm and other components between compartments. Pseudohyphae (2) are less elongated hyphae which are more constricted at septa than true hyphae. Pictures courtesy of Simon Vautier *(4)*.

Candida spp. are normal flora of the human body and they exist as commensals of the skin, mouth and gastrointestinal tract. Their growth and spread are controlled by coexisting microbial flora, intact epithelial barriers, and innate immune system defences (5).

These yeasts possess the ability to act as pathogens that cause a wide spectrum of conditions, ranging from superficial infections to life-threatening systemic infections. The presence of candidemia results in an increase in mortality and hospital length of stay, making *Candida* not only a clinical concern but an economic concern as well (6). It is estimated that fungal diseases, in the US, costed more than $7.2 billion in 2017. Hospitalizations for *Candida* infections (n = 26 735, total cost $1.4 billion) and *Aspergillus* infections (n = 14 820, total cost $1.2 billion) accounted for the highest total hospitalization costs of any disease (7).

The incidence of fungal infections caused by *Candida* spp. has increased dramatically during the last decades. This is mainly due to the rise in number of immunocompromised patients (8). *Candida* infections are derived from the individual's own endogenous reservoir (9) when the host presents certain risk factors such as immunosuppressive and cytotoxic therapies, treatment with broad spectrum antibacterial antibiotics, AIDS, diabetes and drug abuse. *Candida* spp. are responsible for 8% to 10% of nosocomial bloodstream infections (10) and mortality due to systemic candidiasis remains high (46% to 75% for *C. albicans* alone) (11).

There are over 150 heterogeneous species included in the *Candida* genus (12). *C. albicans* is the most common cause of infection, but there have been increased numbers of isolations of non-*albicans* species of *Candida* in recent years, with the most prominent being *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*. These species are the five more common and together they account for 92–95% of all cases of *Candida* infection (13). And while this still holds true, there has been a shift in the species distribution among *Candida* infections. Other species of *Candida* are also emerging, and they are called rare yeasts. This term is used for a category of ascomycetous yeasts that have low incidence (1% of clinical *Candida* infections) and present high minimum inhibitory concentration (MIC) values for at least one class of antifungals. There's lack of susceptibility data thanks

to the limited clinical experience. Consequently, infections caused by these rare fungal pathogens are linked to high mortality and therapeutic failure (14). Examples of these non-*albicans* species are *Candida palmioleophila* and *Candida inconspicua*. The rise in the non-*albicans Candida* spp. is possibly due to their high levels of intrinsic antifungal drug resistance, but it is also possible that improvements to laboratory detection and identification may provide more specific identification than in the past and account for emergence of less common species (6).

1.2.1. *C. albicans*

C. albicans is the most prevalent fungal species of the human microbiota. This species colonizes many areas of the body, particularly the gastrointestinal and genitourinary tracts of healthy individuals (15). It can cause diseases ranging from mucosal to systemic infections. The ability of this highly adaptable yeast to transition from commensal to pathogen is due to a range of virulence factors. Specifically, the ability to switch morphology and form biofilms are properties central to its pathogenesis. In fact, the majority of *C. albicans* infections are associated with biofilm formation on human tissue or abiotic surfaces such as implanted medical devices, which carry high morbidity and mortality (16) (17). Unfortunately, current numbers don't reflect the true extent of damage caused by this yeast given that there have been recent reports indicating that *C. albicans* can cross the blood/brain barrier (18) (19). Nevertheless, given its extensive characterization over the years, *C. albicans* is commonly used as a model organism for microbiology.

1.2.2. *C. glabrata*

C. glabrata is responsible for up to 29% of total *Candida* bloodstream infections (20) (21). It is the second most common bloodstream *Candida* spp. in the US and the Northern Europe (20) (22) (23) and the third or fourth in Asia (24) (23) (25). Bloodstream infections caused by *C. glabrata* are more common in the elderly, diabetic patients and organ transplant recipients. *C. glabrata* can also cause vaginal and urinary tract infections (26) (27). *C. glabrata* is significantly less pathogenic than *C. albicans*. The lack of invasive hyphal forms, secreted proteolytic activity and invasins, and limited nutrient plasticity are likely to contribute to its low pathogenicity (28). The treatment of infections caused by this yeast remains a challenge given that *C. glabrata* presents high levels of intrinsic and acquired resistance to azole antifungals, especially due to overexpression of multidrug resistance transporters (29) (30).

1.2.3. *C. krusei*

C. krusei has been described as a causative agent of disseminated fungal infections in susceptible patients, resulting in the lowest 90-day survival rates (53.6%) among common *Candida* spp. (31). It is a well-known fungal pathogen for patients with hematologic malignancies and for transplant recipients (32). Although the prevalence of *C. krusei* remains low (2%) among yeast infections (31), it attracts much medical attention because it is intrinsically resistant

to fluconazole. In addition, *C. krusei* exhibits resistance to other antifungal drugs such as voriconazole, echinocandins, and amphotericin B (33).

1.2.4. *C. parapsilosis*

C. parapsilosis is an evolving major human pathogen that over the past two decades has increased dramatically in importance and incidence. It is one of the most isolated *Candida* spp. from blood cultures in Europe, Canada and Latin America, and is more prevalent than *C. albicans* in some European hospitals. It has been associated with disease in humans such as endocarditis, meningitis, septicemia, peritonitis, arthritis, endophthalmitis, keratitis, otitis, cystitis, and skin infections. Individuals at the highest risk for severe infection include neonates and patients in intensive care units. Factors involved in disease pathogenesis include the secretion of hydrolytic enzymes, adhesion to prosthetics, and biofilm formation (34).

1.2.5. *C. palmioleophila*

C. palmioleophila, formerly known as *Torulopsis candida*, is an ascomycetous lipolytic yeast related to *Candida famata* and *Candida saitoana* (35). It has been misdiagnosed as *C. famata* or *Candida guilliermondii* but in contrast to these species, *C. palmioleophila* displays a unique susceptibility profile. Available data shows that clinical isolates of *C. palmioleophila* have been shown to be highly susceptible to echinocandins and less susceptible to azoles, especially to fluconazole to which this species has shown resistance (36). As a pathogenic fungus, there are reports of endogenous fungal endophthalmitis by *C. palmioleophila*. Ocular candidiasis complicates candidemia at a rate of approximately 2 to 26% (37).

There are few reports describing the biochemical and physiological characteristics of *C. palmioleophila,* but the available information suggests a high potential of this yeast as bioremediation agent of effluents contaminated with fats and oils. A study reported the isolation and characterization of native strains of *C. palmioleophila*, that showed high lipolytic activity, from solid and liquid wastes of a palm oil refining process. These were capable to successfully assimilate and degrade up to 79% of palm oil (38). Another study also reported that a consortium of several strains of *C. palmioleophila* degraded more efficiently palm oil, up to 84%, than individual strains. The consortium was successfully used for the bioremediation of Palm Oil Mild Effluents (39). The specific enzyme mechanisms and metabolic pathways used by *C. palmioleophila* to degrade oil are not yet described but lipases and esterases are most likely involved in the first steps of this process (35). *C. palmioleophila* may also have a practical application in the biotransformation of various dye effluents because of high decolorizing activity against various azo dyes commonly used in the textile industries (40).

1.2.6. *C. inconspicua*

C. inconspicua is an emerging pathogenic, very rarely isolated from patients. It is phylogenetically related to *Pichia norvegensis* and *C. krusei.* It presents fluconazole resistance and low susceptibility to itraconazole and posaconazole

(14). It has also showed intermediate resistance to flucytosine and *in-vitro* drug susceptibility tests showed susceptibility to amphotericin B (41) (42) (43) (44) (14). Another study has also described *C. inconspicua* as susceptible to echinocandins (45).

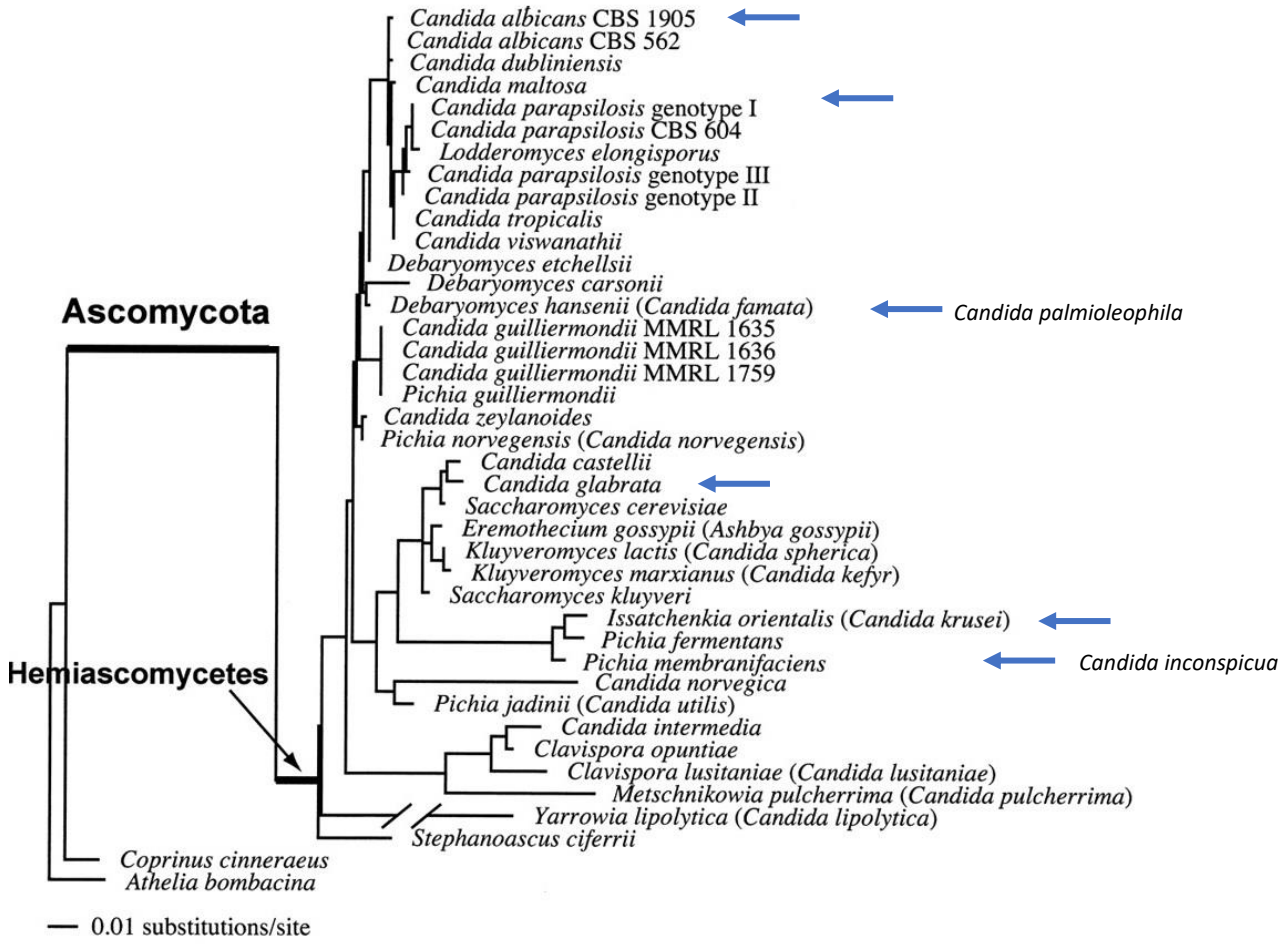


Figure 2 – Phylogenetic tree representing mainly the evolutionary relationships between *Candida* species. Adapted from Stephanie Diezmann et al, 2004. Marked with arrows for species contemplated in this study. *Candida palmioleophila* and *Candida inconspicua* were added and their probable position in the tree is based on the known phylogenetic relations of these species *(46)*.

1.3. Antifungal drugs

Antifungal therapy is a central component of patient management for *Candida* infections. They can be treated using four main drug classes: azoles, polyenes, pyrimidine analogues and echinocandins (Figure 3).

Polyene drugs consist primarily of amphotericin B and nystatin. The primary mode of their antifungal activity results from binding to ergosterol, a component of the cell membrane. This binding forms channels in the cell membrane,

altering its permeability and causing leakage of sodium, potassium, and hydrogen ions, which disrupts the proton gradient and results in cell death. Polyenes also bind to a lesser extent to cholesterol of mammalian plasma membrane, which accounts for most of the toxicity associated with their use (47) (48).

Flucytosine is converted to an antimetabolite, 5-fluorouracil, selectively in fungal cells by cytosine deaminase. This antimetabolite competes with uracil for incorporation into fungal RNA and it is metabolised to compounds that inhibit enzymes involved in DNA synthesis. Selective toxicity for fungus is achieved with flucytosine because mammalian cells do not take up the drug or convert it to 5-fluorouracil. Flucytosine is normally used in combination with amphotericin B, which appears to increase fungal uptake of flucytosine and result in synergistic effects against certain fungal diseases. The use of flucytosine can be toxic at a hepatic level which can many times limit its use (49).

Allylamines inhibit the enzyme squalene epoxidase which is important in the synthesis of ergosterol. The accumulation of squalene turns the cell membrane deficient in ergosterol. The resulting ergosterol depletion and toxic squalene accumulation affect cell membrane structure and function, eventually leading to inhibition of growth and to cell death (50).

Azoles are one of the most broadly used antifungal drug classes and inhibit ergosterol biosynthesis by targeting lanosterol 14-α-sterol demethylase, encoded by the *ERG11* gene in yeasts. This enzyme participates in the conversion of lanosterol to ergosterol, the latter being involved in the cell membrane integrity. By inhibiting the lanosterol 14-α-sterol demethylase, azoles induce the accumulation of a toxic sterol compound, disturbing the cell membrane, leading to growth inhibition and cell death (51).

Echinocandins are recommended by the Infectious Diseases Society of America and the European Society for Clinical Microbiology and Infectious Diseases as the first-line empirical treatment for invasive candidiasis. They are in widespread clinical use because of the efficacy demonstrated by this antifungal agent (52). Echinocandins specifically inhibit the biosynthesis of the fungal-specific enzyme (1,3)-β-D-glucan synthase, and (1,3)-β-D-glucan is a major structural component of fungal cell walls. This enzyme inhibition leads to the formation of fungal cell walls with impaired structural integrity, which finally results in cell vulnerability to osmotic lysis. All three agents, caspofungin (CSP), micafungin (MCF), and anidulafungin (AND), exhibit concentration-dependent fungicidal activity against most species of *Candida* (Figure 4). These antifungals have generally favourable safety and tolerability profiles with adequate pharmacokinetics and few drug interactions. The use of echinocandins for prophylaxis and treatment has been expanding, and more than 60% of candidemia patients are now reported to receive an echinocandin (53).

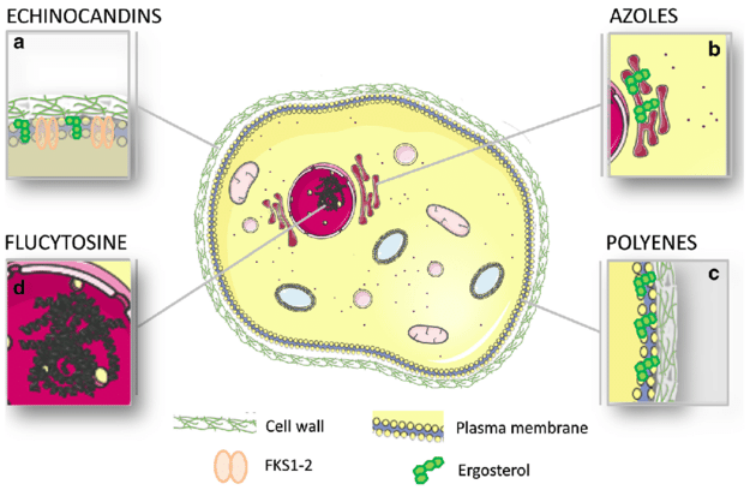


Figure 3 – Adapted from Danièle Maubon et al, 2014. Targets and mechanisms of action of systemic antifungal drugs. Sites and modes of action of the current classes of systemic antifungal drugs used to treat invasive candidiasis. a) Echinocandins target cell wall synthesis, inhibiting (1,3)-β-D-glucan synthesis, which occurs on the inner side of the plasma membrane. b) Azoles target the ergosterol biosynthesis pathway in the endoplasmic reticulum. They block 14-α-demethylase, resulting in ergosterol depletion in the membrane and activation of the Erg3p alternative pathway, leading to the synthesis of toxic sterols. c) Polyenes bind to cell membrane ergosterol creating pores and aggregate, to act as a ''sponge'', thus resulting in ion depletion. d) Flucytosine acts in the nucleus, where its toxic metabolites inhibit nucleic acid synthesis *(54)*.

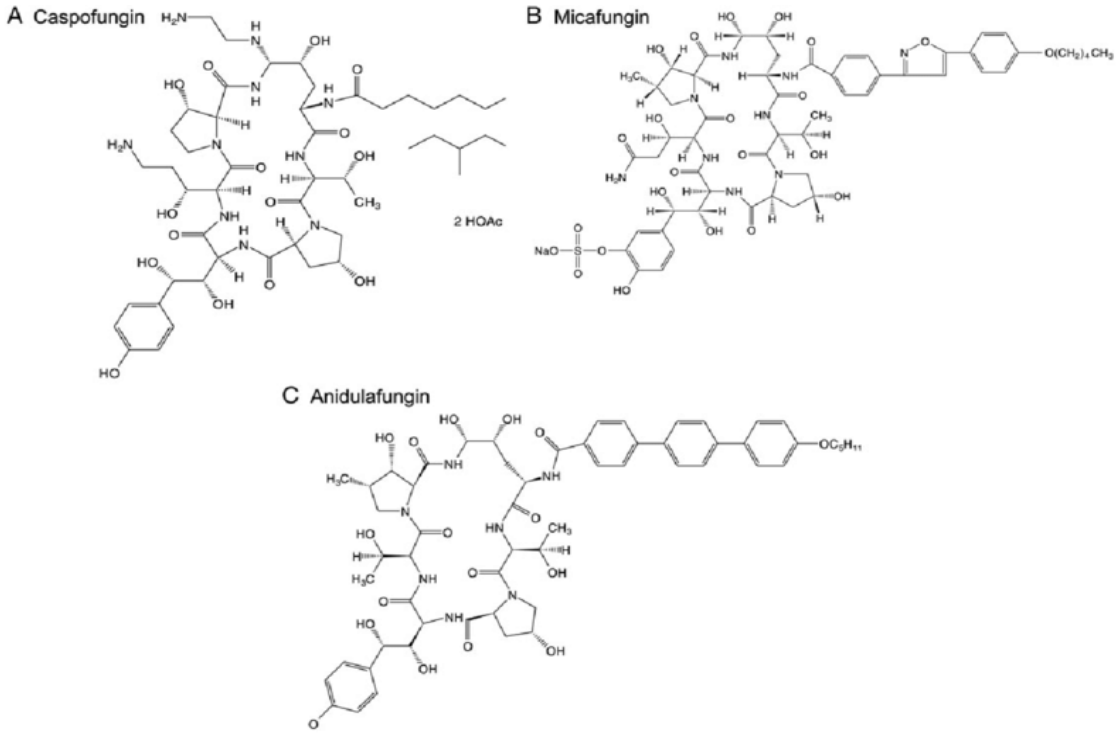


Figure 4 – Adapted from Daniel Aguilar-Zapata et al, 2015. Chemical structure of echinocandins. A) Caspofungin: $C_{52}H_{88}N_{10}O_{15}$ × 2 $C_2H_4O_2$; molecular weight = 1213.4. B) Micafungin: $C_{56}H_{71}N_9O_{23}S$; molecular weight = 1292.26. C) Anidulafungin: $C_{58}H_{73}N_7O_{17}$; molecular weight = 1140.2 *(55)*.

1.3.1. Antifungal susceptibility

High rates of morbidity and mortality associated with fungal infections have to do with the current limited antifungal agents and the high toxicity of those compounds. Since there are many similarities between fungal and human cells it is difficult to identify novel drug targets (56). Not only this but treatment of yeast infections is further compromised by the rise of antifungal drug resistance. Evolution of multidrug resistant organisms is the most concerning, especially among common *Candida* species. Therapeutic failure relates to clinical resistance in which, at a normal dose, a patient fails to react to an antifungal agent. Antifungal resistance development is complex and is dependent on numerous host and yeast variables (57). The host immune system status is a critical factor because antifungal drugs must operate synergistically to regulate and clear an infection. The likeliness of therapeutic failure is higher for patients with severe immune dysfunction due to the fact that the antifungal drug must act alone without an immune system response (58). The existence of medical and surgical devices can also lead to resistant infections, as infectious organisms can create biofilms by attaching themselves to these objects and therefore withstand drug action. To guarantee an appropriate treatment it is necessary that each drug reaches the infection site at the appropriate concentration that is sufficient for antifungal action. Although the pharmacokinetics of many drugs are characterized there is limited scientific data of drug penetration in all sites of infection. Some microorganisms are therefore subjected to drugs at suboptimal levels and this leads to cells that persist during treatment. This results in endogenous reservoirs that originate new infections. All these factors contribute to resistance, which refers to the selection of strains that can proliferate despite exposure to therapeutic levels of antifungals. During treatment, such strains contribute considerably to drug failure (59) (60) (61). Resistant strains can be divided in two categories, they are either primary resistant strains that are inherently less prone to a specified antifungal agent or secondary resistant strains that attain a resistance feature following drug exposure in an otherwise sensitive strain. The molecular mechanisms involved in secondary resistance are often expressed at various levels in primary resistant strains (62).

To determine the occurrence of resistance to antifungal agents, the quantification of antifungal susceptibility has been standardized using different protocols. Antifungal susceptibility testing (AFST) is performed on yeast causing disease, especially if the infection is invasive, relapsing or failing therapy, when inherent or acquired resistance is a possibility or when susceptibility cannot reliably be predicted from the species identification alone. AFST is also important for resistance surveillance, epidemiological studies and for comparison of the in vitro activity of new and existing agents (63). MICs are defined as the lowest drug concentration resulting in a significant reduction of growth (usually either 50% or 90% reduction compared with growth in the absence of the drug). This is the reference method for AFST, and its mainly used to: establish the activity of new antifungal agents, confirm the susceptibility of organisms that give equivocal results in other test formats and to determine the susceptibility of organisms where other test formats may be unreliable or not yet validated. Data from these studies has been used to determine MIC breakpoints, which are MICs at which an organism should be considered susceptible, intermediate, or resistant in relation to a certain antifungal (64). Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI), have standardized methods to perform AFST. Both institutions have developed breakpoints of some antifungals to *Candida* spp. that are currently used to classify

resistant strains. Testing can also be carried out using Etest manufacturer's guide (65). A yeast is classified as: susceptible when the it is inhibited in vitro by a concentration of the tested drug that is associated with a high likelihood of therapeutic success; intermediate when it is inhibited in vitro by a concentration of the tested drug that is associated with an uncertain therapeutic effect; resistant when it is inhibited in vitro by a concentration of the tested drug that is associated with a high likelihood of therapeutic failure (66).

Challenges with patient treatment are proportional with developing resistance and therefore rapid identification of resistance and its mechanisms is necessary. Once identified, treatment can be more adequately adjusted to the patient's needs (51).

1.3.2. Mechanisms of antifungal resistance

Various mechanisms of antifungal resistance have been identified and for the most part they are involved with reduced intracellular drug accumulation, counter action of the drug effect and decreased target affinity. The presence of these mechanisms depends on the mode of action of the antifungals.

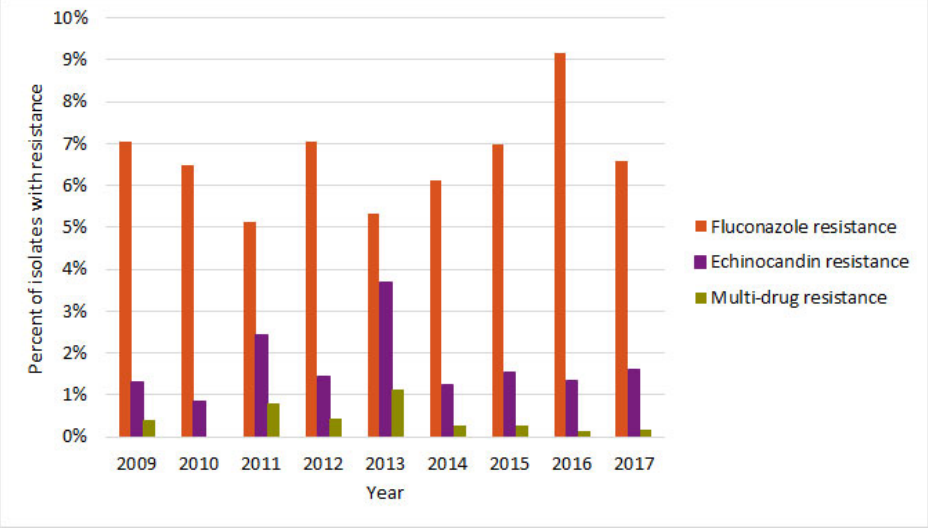


Figure 5 – Adapted from Centres for Disease Control and Prevention. Antifungal resistant *Candida* species isolates in the US, by year, 2009–2017 *(67)*.

Resistance mechanisms against azole antifungal agents are the most documented in *Candida* spp. (Figure 5). One of the mechanisms that causes the decrease in intracellular concentration of azole is the upregulation of two families of efflux pumps. These two families have two different sources of energy needed to pump out the drug and differ in the specificity of the azole molecule. The superfamily of ATP-binding cassette (ABC) transporters has the *Cdr* pumps encoded by *Candida* drug resistance 1 (*CDR1*) and 2 (*CDR2*) genes in *C. albicans*. The major facilitator superfamily (MFS) has a pump that is a secondary transporter and uses as a source of energy the proton gradient. This pump is encoded by the *MDR1* gene in *C. albicans* (68). Mutations on the *TC1* and *MRR1* transcription factors are responsible, respectively, for the upregulation of *CDR1*/*CDR2* and *MDR1* (69) (70). Other transporter genes have

been reported to be upregulated in azole-resistant *C. glabrata* (*CgCDR1*, *CgCDR2*, *CgSNQ2*) (71) (72) (73), *C. krusei* (*ABC1*, *ABC2*) (74) (75)*, Candida dubliniensis* (*CdCDR1*, *CdCDR2*) (76) and *C. tropicalis* (*CDR1*-homologue) isolates (77). In *C. glabrata*, *CgCDR1*, *CgCDR2*, and *CgSNQ2* genes are regulated by the *CgPDR1* transcription factor (78) (79) (80).

The target of azole antifungals is lanosterol 14-α-demethylase encoded by the *ERG11* gene. Several point mutations on this gene have been characterized and associated to high azole MICs and they contribute for the decreased affinity between the drug and the target. There are also two mechanisms associated with counteracting the drug effects of azoles. One of them is the upregulation of the *ERG11* gene caused by the regulation of transcription factors and gene duplication. This leads to an intracellular increase of the target protein. The other mechanism is very uncommon, but it has been identified in several clinical isolates of *C. albicans* (81). The C5 sterol desaturase gene (*ERG3*) is essential for ergosterol biosynthesis. Point mutation in *ERG3* lead to the inactivation of the gene and total inactivation of C5 sterol desaturase, interfering with the ergosterol biosynthesis pathway. In this case, there is no longer an accumulation of 14-α-methylated sterols and the cell membranes produced are lacking ergosterol (82) (Figure 6).

There is evidence of counteraction of drug effect when it comes to polyenes. Reports show that *Candida* isolates that are polyene-resistant, when compared with susceptible isolates, show a decrease in ergosterol content. Loss of function mutations in the *ERG3* and *ERG6* genes are probably responsible for this phenomenon given that these genes encode enzymes that are involved in the biosynthesis of ergosterol. Therefore, there is a substitution of ergosterol by other sterols (83) (84) (85).

Primary resistance to flucytosine remains low (<2%). Secondary resistance is consequence of the inactivation of different enzymes of the pyrimidine pathway. Point mutations in the *FCY2* gene contribute to reduced intracellular accumulation of flucytosine because this gene encodes the cytosine permease, part of the pyrimidine pathway. Point mutations in the *FCY1* or *FUR1* (most frequent) gene can also result in acquired resistance to flucytosine by counteraction of the drug effect. *FCY1* encodes the cytosine deaminase and *FUR1* encodes the uracil phosphoribosyl transferase. These enzymes are catalysts in the conversion of 5-fluorocytosine to 5-fluorouracil and 5-fluorouracil to 5-fluorouridine monophosphate, respectively. Alterations in expression lead to interference in the pyrimidine pathway by the decreased synthesis of the active nucleotide metabolites. Several point mutations have been described in *C. albicans*, *C. glabrata*, and *C. lusitaniae* (86) (83) (87) (88).

Resistance to allylamines is rare but may occur owing to enhanced efflux pump activity or mutations in squalene epoxidase (89).

1.3.3. Echinocandin resistance mechanisms

Resistance to echinocandin antifungal agents was first reported in 2005 (90) and remains relatively low, at <3% with *C. albicans* and most *Candida* spp. (91). The exception is *C. glabrata*, in which echinocandin resistance is rising and there is cause for alarm as many isolates show cross-resistance to azole antifungal agents (92) (93) (94).

Unlike azole antifungal agents, resistance to echinocandins is largely unaffected by multidrug transporters (95) (96). In *Candida* spp., resistance mutations occur in two highly conserved hot spot regions of *FKS* gene product, *FKS1* and *FKS2*, the catalytic subunit of (1,3)-β-D-glucan synthase (Figure 6). The amino acid substitutions decrease sensitivity of glucan synthase to drug by 50 to 3000-fold (97) (98), and elevate MIC values 5 to 100-fold (99) (100). The echinocandin resistance level conferred by hot spot mutations in *FKS1* or *FKS2* may also depend on the relative expression of these genes, which can vary more than 20-fold (98) (101).

Inhibition of glucan synthase by echinocandins causes cellular stress and yeasts possess adaptive cellular factors in order to provide protection against these stresses (102) (103). Drug tolerance may be an important intermediate stage for development of resistance. Consistent with this model, fungal stress tolerance pathways including cell wall integrity, enable survival of echinocandin induced stress by controlling compensatory upregulation of chitin synthesis (104) (105). In treatment with echinocandins, there are many changes in cell wall composition that occur in *Candida* spp., not only the increase in cell wall chitin content, but also the decrease in β-glucans and the upregulation of cell surface proteins (106). The cell wall integrity circuitry is not only crucial for responses to cell wall stress induced by echinocandins, but it is also required for cell membrane stress induced by azoles (107), highlighting its central role in mediating antifungal drug resistance.

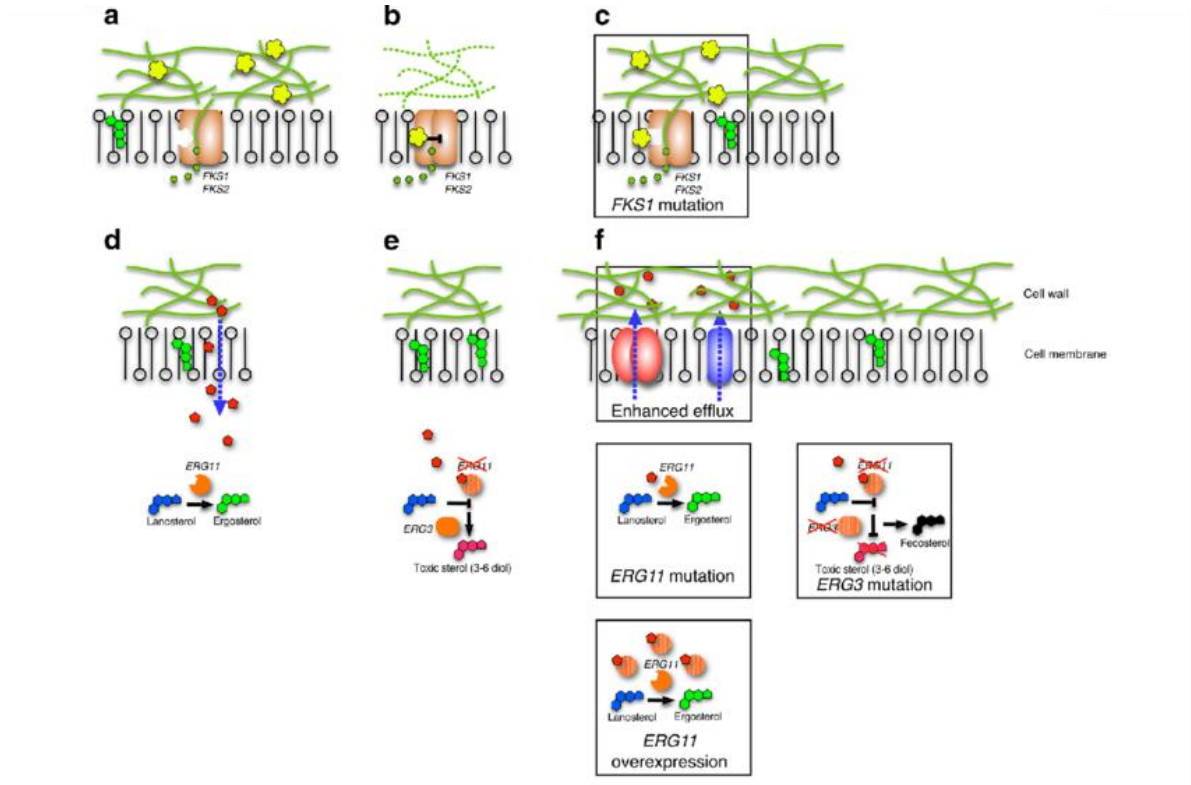


Figure 6 – Adapted from Danièle Maubon et al, 2014. Molecular mechanisms of echinocandin and azole resistance in *Candida* species a) Regular (1,3)-β-glucan synthesis on the inner side of the fungal membrane. b) Typical echinocandin activity. These compounds block cell wall synthesis by inhibiting the FKS subunit of the (1,3)-β-glucan synthase. c) Echinocandin resistance due to FKS mutations. The target enzyme is less sensitive to echinocandins, allowing the production of (1,3)-β-glucans. d)

Typical ergosterol synthesis at the endoplasmic reticulum and uptake of azole antifungal drugs into the cytosol of the fungal cell. e) Typical azole activity. These molecules inhibit the lanosterol-14a-demethylase (Erg11p), leading to (1) membrane ergosterol depletion and (2) the production of toxic sterols via Erg3p. f) Azole resistance due to (1) the overproduction of transporters, increasing azole efflux, (2) alteration of the target enzyme by mutations of ERG11, (3) Erg11p overproduction, (4) mutations of ERG3 preventing the azole mediated production of toxic sterols which are substituted by the non-toxic fecosterol *(54)*.

1.4. Susceptibility of *Candida* spp. in Poland

Little is known on the epidemiology of *Candida* spp. in Poland. Nevertheless, the frequency of infections caused by non-*albicans* species is increasing. The prevalence of non-*albicans* species increased from 12.5% to 70% in ten years. The same report showed that for 118 clinical cases of *Candida* infections in polish hospitals the mortality rate was 8.5%. *C. albicans* and *C. parapsilosis* were the most prevalent species (39.8% and 35.6% respectively) and fluconazole had the highest resistance rate of 7.6% (108). The most common antifungal drug used in polish intensive care units (ICUs) is fluconazole, accounting for 97% of all the antifungal drugs (109).

Regarding the distribution of *Candida* spp. in Poland, a total of 302 cases of candidemia were reported over two years in 20 polish hospitals. *C. albicans* was the most isolated species, accounting for 50.96% of isolates, followed by *C. glabrata* and *C. parapsilosis* with frequencies of 14.10% and 13.14% respectively, and the distribution of *C. tropicalis* and *C. krusei* was at 6.73% and 6.41%. Other Candida spp. (*C. famata, C. dubliniensis, Candida sake, Candida kefyr, Candida lusitaniae, C. quilliermondii, C. inconspicua*) comprised of 8.65% of the isolates (110).

Reports on resistance of *Candida* spp. in Poland are scarce, especially regarding echinocandins. There are two reports from 2008 on caspofungin susceptibility testing performed with Etests on isolates collected in polish hospitals and the examined *Candida* isolates were susceptible to echinocandins (111) (112). Another three reports from 2012 (113), 2014 (114) and 2015 (115) had similar results. In 2015 a study performed in the Medical University of Lublin in Poland presented data that showed that most of the studied clinical isolates – 27 (90%) showed sensitivity to micafungin, with MIC values ranging from 0.004 to 2 mg/L, while 3 (10%) isolates, including 2 isolates of *C. tropicalis* and 1 isolate of *C. famata*, were resistant to micafungin, with MIC values > 32 mg/L (116).

1.5. Cell wall of *Candida* spp. as a target for antifungal research

The cell wall is an essential and highly dynamic fungal structure that has been implicated in several physiological processes. These include the maintenance of cellular morphology and osmotic protection of the cell through its rigidity. The cell wall can modulate the immunological response against infection. To better understand at molecular level the organization of the fungal cell once drug resistance has been established, cell wall represents a first choice as this compartment constitutes her barrier between the yeast the and host. In addition, its absence in mammalian cells makes it an ideally attractive target in antifungal research (117).

Studies for identification of cell wall proteins (CWPs) that change their expression in resistant strains may be useful to determine biological markers associated to drug resistance. Furthermore, determination of the functional properties of differently expressed CWPs may highlight metabolic pathways involved in sustaining the virulence of yeasts. A suitable strategy to study modulation of cell wall proteins in resistant strains and clinical isolates is the analysis of gene expression that can give a better picture of the differential expressed proteins between sensitive and resistant strains.

1.5.1. *FKS* gene and upregulation of genes encoding cell wall proteins

As previously mentioned, *FKS1* and *FKS2* encode catalytic subunits of the glucan synthases that are responsible for synthesis of (1,3)-β-glucan in the cell wall. Reports describe that in *Saccharomyces cerevisiae* the deletion mutant *fks1*Δ reduces the glucan content of the cell wall and this results in an increase in the chitin content (also described for *Candida* spp. (118)), upregulation of the *PST1* gene and activation of the expression of *CWP1*, which encodes glycosylphosphatidylinositol (GPI)-dependent cell wall protein (119) (120). The induction of transcription of *CWP2* gene occurs around the same time as *CWP1* and might therefore be brought about by similar mechanisms (121). These cellular responses have been regarded as compensating for cell wall damage in order to maintain cell wall integrity. The cell wall model suggests that the three cell wall components, glucan, chitin and mannoproteins, are bound to each other and form a large complex to ensure cell wall integrity. The decrease in (1,3)-β-glucan synthesis could be compensated for by changing the type of association among them.

CHS1 gene encodes for chitin synthase 1, located in the plasma membrane, and it is involved in the formation and repair of the disk-shaped septum in yeast (122). Chitin plays important roles in maintaining the mechanical strength of the fungal cell wall, thus keeping its integrity (123). Damage to the cell wall may be countered by the elevated quantity of chitin in cell wall due to increased synthesis and/or decreased degradation of chitin, which may increase the tolerance to antifungal drugs (124). Because this gene is essential *in vitro* and *in vivo* and is not present in humans, it represents an attractive target for the development of antifungal compounds (125).

PST1 gene encodes for a GPI-modified protein (homologous to a protein encoded by *ECM33*), covalently incorporated in cell wall fraction of exponential-phase cells (126). The function of the encoded protein is still unknown but it was reported that its upregulated by cell wall damage and by the cell integrity pathway (127). A homologous gene to *PST1* has been reported in *C. albicans* (128).

Cell wall proteins (CWP) play key roles in cell wall assembly, adhesion to host surfaces, as immunomodulators and in protecting the fungus from host enzymes. Alterations in the cell wall proteome would introduce variation at the yeast surface, and such changes could enhance the ability of the fungus to evade the host's immune system and to colonize different sites of the body (129). *CWP1* and *CWP2* genes encode two major (GPI)-anchored mannoproteins of the cell wall (130). Cells have the ability to attach these CWPs to the wall either via linkages to (1,3)-β-glucan, (1,6)-β-glucan or both (131).

2. Materials and Methods

2.1. Strains

A total of 78 *Candida* strains belonging to six different species (48 *C. albicans*, 18 *C. glabrata*, 5. *C. palmioleophila*, 3 *C. krusei*, 3 *C. parapsilosis*, 1 *C. inconspicua*) were used during this set of experiments. These strains were isolated, between the years of 2008 to 2012, from patients of four polish hospitals: Children's Memorial Health Institute in Warsaw (CZD), Medical University of Gdansk, Pomeranian Medical University in Szczecin and Wrocław Medical University. The isolates originated from a variety of clinical specimens, isolated from swabs of the mouth, throat, faeces, urine, blood, and bronchopulmonary lavage fluid.
The strains used for each set of experiments were chosen before any assay took place.

2.2. Antifungal susceptibility assays in *Candida* spp.

A total of 30 *Candida* species (21 *C. albicans*, 2 *C. glabrata*, 1 C. *inconspicua*, 1 *C. krusei*, 5 *C. palmioleophila*) were submitted to ASFT.

Broth microdilution methods are used to establish the MICs of antimicrobial agents. In dilution tests, yeasts are evaluated for their ability to produce sufficient growth in microdilution plate wells of broth culture media containing serial dilutions of the antimicrobial agents. MICs show the in vitro activity of a given antifungal drug under the described test conditions. The MIC also allows fungi to be categorised as susceptible (S), intermediate (I), or resistant (R) to an antifungal drug when appropriate breakpoints have been established.

All antifungal drug solutions were prepared in accordance with Good Manufacturing Practice. The echinocandins powder was obtained from the following manufactures: Pfizer (AND), Sigma-Aldrich (CSP) and Astellas (MCF).

2.2.1. Medium

RPMI 1640 (with L-glutamine and a pH indicator but without bicarbonate) supplemented with glucose to a final concentration of 0.2% (RPMI 0.2% G) was used. Buffer 3-(N-morpholino) propane sulfonic acid (MOPS) was used to obtain a pH of 7.0 at 25^{o}C. The mixture was filtered. For quality control purposes, the sterilised medium without the antifungal was used for sterility checks, for retesting the pH and as a growth control with a reference strain.

2.2.2. Stock solutions

Antifungal drug solutions must be prepared considering the potency of the lot of antifungal drug powder that is being used. The amount of powder or diluent required to prepare a standard solution may be calculated as demonstrated by equations (1) and (2).

$$\text{Weight (g)} = \frac{\text{Volume (L)} \times \text{Concentration} \left(\frac{\text{mg}}{\text{L}}\right)}{\text{Potency} \left(\frac{\text{mg}}{\text{g}}\right)} \quad (1)$$

$$\text{Volume (L)} = \frac{\text{Weight (g)} \times \text{Potency} \left(\frac{\text{mg}}{\text{g}}\right)}{\text{Concentration} \left(\frac{\text{mg}}{\text{L}}\right)} \quad (2)$$

The hydrophobic solvent used for echinocandins was dimethyl sulfoxide (DMSO). It was essential to ensure the drug was fully dissolved given that several antifungals can be difficult to dissolve resulting in artificially elevated MICs.

2.2.3. Working solutions

The range of concentrations tested encompassed the breakpoint as well as the expected results for the quality control strains. The drug concentration ranges recommended are 0.008 – 4 mg/L for the three echinocandins.

2.2.4. Microdilution plates

The microdilution wells were disposable and composed of sterile plastic since high binding plastic had to be avoided. The series of dilutions started at 100 times the final concentration and after each working solution was diluted 10 times in the RPMI-1640 broth.

2.2.5. Inoculation of microdilution plates

The inoculum was prepared by suspending representative colonies of the isolates, obtained from an 24 h culture at approximately 35^{o}C on Sabouraud's dextrose agar, in saline solution. The final inoculum was around 2.5 x 10^5 CFU/mL. The cell density was adjusted to the density of a 0.5 McFarland standard by measuring absorbance in a spectrophotometer at a wavelength of 530 nm.

Each well of the microdilution plate was inoculated with yeast suspension. The growth control wells were also inoculated, containing sterile drug-free medium and inoculum suspension. One well contained saline solution from the lot used to prepare the inoculum as a sterility control for medium and distilled water (drug-free medium only). Quality control organisms were tested by the same method each time an isolate was tested.

Viability counts were performed for quality control purposes to ensure that test wells contained between 1 – 5 x 10^5 CFU/mL. The suspension was homogenized, and a sample streaked over the surface of a Sabouraud dextrose plate, which was then incubated.

2.2.6. Incubation and reading results of microdilution plates

Microdilution plates were incubated without agitation at 35^{o}C for approximately 24 h. The growth on each plate containing the antifungal agent was visually compared with the growth on the control plate (without the antifungal for each yeast). MIC values were determined visually after 24 h of incubation as the lowest concentration of drug that caused a complete growth inhibition.

2.2.7. Quality control

MICs for control strains should ideally be close to the middle of the range of the two-fold series tested and antifungal drug susceptibility patterns must be stable. The control strains were selected according to these criteria, *C. albicans* ATCC 90028 and *C. krusei* ATCC 6258.

2.3. Genotyping and PCR fingerprinting

Genotyping is the process of determining differences in the genotype of an individual by examining the individual's DNA sequence using biological assays and comparing it with another individual's sequence or a reference sequence (132). One of the various techniques used is genotyping with Polymerase Chain Reaction (PCR). The advantages of using PCR include much faster results and not having to handle radioactivity (133).

The high similarity between yeast species causes significant problems in the correct identification in a standard clinical mycology laboratory. PCR fingerprinting was performed to distinguish between clinical isolates of closely related species (134). A single primer was used in each PCR assay to amplify DNA sequences from 70 isolates (40 *C. albicans*, 18 *C. glabrata*, 5 *C. palmioleophila*, 3. *C. parapsilosis*, 3 *C. krusei*, 1 *C. inconspicua*). Three different single primers were tested: A, B and C (Table 1).

Table 1 - Primer A (*TRP* gene), B (*HSP* gene) and C (*FKS* gene) sequences used for PCR fingerprinting.

Primer	Gene	Sequence (5' to 3')
A	*TRP*	AATTGTTCCAGCGTTTTTGT
B	*HSP*	TGCAACCACAAGAGGCTTAAC
C	*FKS*	TTGACTTTGTCTTTAAGATCC

The goal was that each species could be identified by a distinct species-specific multilocus pattern, allowing species identification for all clinical isolates. In addition, the PCR fingerprinting generates strain specific profiles, making this method applicable to epidemiological investigations.

2.3.1. DNA isolation

In order to perform genotyping by PCR for *Candida* spp. it was first needed to extract and isolate the DNA from the microorganisms. DNA extraction was performed by taking a small fragment of mycelium and resuspending it in 100 µl of extraction buffer (60 mM $NaHCO_3$, 250 mM KCl and 50 mM Tris buffer, pH 9.5), followed by 10 min incubation at 95°C. Next, 100 µl of neutralization buffer was added (2% bovine serum albumin). After vortex mixing DNA-containing solution was stored at 4°C for subsequent analysis (135).

2.3.2. PCR reaction

The components needed to perform the PCR reaction were the following: 10 µL of PCR Mix (A&A Biotechnology, Poland), 0.2 µL of primer, 7.8 µL of sterile water and 2 µL of DNA. The total volume of solution was 20 µL. PCR Mix is an optimized ready to use standard PCR mixture containing High Fidelity Taq DNA polymerase, PCR buffer, $MgCl_2$ and dNTPs. Mix also contains red dye and loading buffer. These additives enable direct loading of PCR products on agarose gel upon completing the PCR. Thermocycler GeneAmp PCR System 2400 (Perkin Elmer) was used for the PCR reaction. The PCR stages and conditions are described in Table 2.

PCR products were detected on 1.5 % agarose gel stained with ethidium bromide, a DNA-binding dye. Optimal conditions for electrophoresis were 110 V for 1 h. DNA fragments of different sizes formed bands on the gel which was visible under UV light.

When comparing the different species patterns on the gel, one of the goals was to identify the bands from non-*albicans Candida* that stand out or differ from the *C. albicans* pattern. To better understand the relation between these patterns and the phylogenetic relations between species, it was necessary to clone and sequence the interest bands. These bands might represent genes of interest for the non-*albicans* species and reveal some new and interesting information.

PCR reaction was repeated for the chosen strains with bands of interest and 60 µL of PCR product was used to perform gel electrophoresis. The bands of interest were cut from the gel and the DNA material isolated using the Gel-Out kit (A&A Biotechnology, Poland). After, NeqSSB polymerase, a polymerase with correction ability, was used to remove AAA adducts from the ends of the PCR product (3 min, 72°C, 0.5 µL of polymerase, 25 µL of product, 3 µL of buffer, 2 µL of water). The DNA was then purified, using the Clean Up kit (A&A Biotechnology, Poland).

Table 2 – Stages of PCR and respective temperature (°C), hold (s), number of cycles and brief description.

Stages	Temperature (°C)	Hold (s)	Cycles	Description
Initialization	95	300	-	The initiation step heats the double stranded DNA template strand to the point where the strands start denaturing and the hydrogen bonds are broken between the nucleotide base pairs.
Denaturation	95	45		Intense heating to denature the DNA strands. This provides single-stranded template for the next step.
Annealing	40	120	35	Cooling of the reaction so the primers can bind to their complementary sequences on the single-stranded template DNA.
Extension	72	120		The temperature is increased so that Taq polymerase extends the primers, synthesizing new strands of DNA.
Final Elongation	72	600	-	A final extension is needed to fill-in any protruding ends of the newly synthesized strands.

2.3.3. Medium

Lysogeny Broth (LB), also known as LB medium, is a commonly used nutritionally rich medium for culturing bacteria. A litre of medium consists of 10 g of tryptone, 5 g of yeast extract, and 10 g of sodium chloride. LB medium is widely used in molecular biology for growing recombinant *Escherichia coli* for use in plasmid purification and protein expression.

LB agar (LA) is LB broth that contains agar and is typically prepared in petri dishes. The flat, solid properties of LB agar allow the plating of bacterial cultures and generation of colonies. Agar is typically added to liquid LB, then autoclaved to produce a hot, liquid solution. A litre of medium consists of 15 g of agar, 10 g of tryptone, 10 g of sodium chloride and 5 g of yeast extract.

2.3.4. Production and purification of the pUC19 plasmid

The cloning vector chosen to carry the genes was pUC19. It is a small, high-copy number *E. coli* plasmid cloning vector with multiple cloning sites. The microorganism used to obtain the plasmid was *E. coli* TOP10. It is a strain of *E. coli* used for cloning and plasmid preparation. It has a high transformation efficiency and it contains the genes for recA, a recombinase, and endA, a DNAse that lowers plasmid yield, taken out.

The *E. coli* TOP10 cells were inoculated on 3 mL of LB for 24 h at 37°C. After, 1 mL of the overnight culture was inoculated with 50 mL LB at 37°C until an optical density at 600 nm of 0.2 (OD_{600}=0.2) was reached. The mixture was centrifuged at 4000 rpm for 10 min and the resultant cell pellet was resuspended in 50 mL of 100 mM of a solution of $CaCl_2$ and incubated on ice for 1 h.

A volume of 1 µL of pUC19 solution was taken and added to 100 µL of competent cells of *E. coli* TOP10. The plasmid-cell mixture was heated (heat shock) at 37°C for 10 min, allowing the plasmid to enter the cell through the disrupted membrane. The heated mixture was then placed back on ice for 2 min to retain the plasmids inside the bacteria. LB medium was then added, 1 mL, and the mixture was incubated for 1 h at 37°C. Finally, 100 µL of the mixture was spread onto plates containing LA medium with ampicillin (AMP), isopropyl-β-D-thiogalactoside (IPTG) and X-gal. The cells were regrown by inoculation of 3 mL of LB containing AMP during 24 h at 37°C. AMP was used in the medium because the pUC19 plasmid encodes an ampicillin resistance gene. The mixture was centrifuged and the plasmid isolated and purified using the Plasmid Mini kit (A&A Biotechnology, Poland). The plasmid was then digested by taking 20 µL of the purified plasmid solution (50 ng/µL) and adding 1 µL of the restriction enzyme SmaI, 3 µL of Tango buffer and 6 µL of sterile water. The mixture was incubated for 1 h at 37°C.

2.3.5. Ligation and transformation

The ligation between the plasmid and gene is one of the crucial steps for a successful cloning. In a tube it was added 20 µL of the digested plasmid, 20 µL of PCR product, 1 µL of ligase, 5 µL of buffer and 5 µL of ATP. The mixture was incubated at 17°C for 1 h.

New competent *E. coli* cells were prepared to proceed with the plasmid transformation. The procedure was to inoculate 20 mL of LB containing tetracycline (TET) with *E. coli* TOP10 F' cells (these contain a gene for TET resistance) and incubate for 24 h. 2 mL of this culture were incubated for 2 hours in a total volume of 50 mL of LB. After, the mixture was centrifuged twice. $CaCl_2$ was added, and the mixture was left to incubate on ice for 1 h. For the transformation of the plasmid containing the gene into the *E. coli* competent cells, 50 µL of pUC19 with insert solution was taken and added to 100 µL of *E. coli* competent cells. The cells were incubated with the ligation mixture for 1 h on ice. A heat shock followed with a duration of 10 min and at a temperature of 37°C. The tube was put on ice for 2 min. 1 mL of LB medium was added and the mixture was incubated for 1 h at 37°C.

The exact mechanism of the uptake of DNA is not known. However, it is thought that the presence of calcium ions allows the negatively charged DNA to come in close contact with the similarly charged cell membrane by acting as the cross bridge. The heat shock step of transformation transiently melts the membrane to complete the uptake process. The efficiency of transformation depends upon many different factors including the competent state of the cells and the properties of the DNA to be transformed.

2.3.6. Cloning and sequencing

In plates with LA medium, AMP, TET, IPTG and X-gal, 150 µL of the bacterial culture was spread and incubated for 24 h at 37°C. For control purposes there was also positive and negative control plates. The negative control was composed of only competent cells with no plasmid to make sure there is no contamination and the positive control contained competent cells with the pUC19 plasmid but with no insert, after growing in LB medium.

The blue-white screen is a screening technique that allows for the detection of successful ligations in vector-based gene cloning. The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white; if not, the colony will be blue. This technique allows for the quick and easy detection of successful ligation.

The host *E. coli* strain carries the lacZ deletion mutant (lacZΔM15) which contains the ω-peptide, while the vectors used carry the lacZα sequence which encodes the first 59 residues of β-galactosidase, the α-peptide. Neither are functional by themselves. However, when the two peptides are expressed together, as when a vector containing the lacZα sequence is transformed into a lacZΔM15 cells, they form a functional β-galactosidase enzyme. The pUC19 vector carries within the lacZα sequence an internal multiple cloning site (MCS). This MCS within the lacZα sequence can be cut by restriction enzymes so that the foreign DNA may be inserted within the lacZα gene, thereby disrupting the gene and thus production of α-peptide. Consequently, in cells containing the vector with an insert, no functional β-galactosidase may be formed. The presence of an active β-galactosidase can be detected by X-gal within the agar plate. X-gal is cleaved by β-galactosidase to form 5-bromo-4-chloro-indoxyl, which then spontaneously dimerizes and oxidizes to form a bright blue insoluble pigment 5,5'-dibromo-4,4'-dichloro-indigo. This results in a characteristic blue colour in cells containing a functional β-galactosidase. Blue colonies therefore show that they may contain a vector with an uninterrupted lacZα (therefore no insert), while white colonies, where X-gal is not hydrolysed, indicate the presence of an insert. Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analogue of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.

After the selection of the white colonies these were re-plated using the line technique, in LA medium, and left to grow overnight. LB medium was then prepared with 100 mL of TET and AMP. A sample of the colonies was mixed with 5 mL of LB and left to grow overnight. The overnight culture was subjected to centrifugation at 4000 rpm for 10 min. The Plasmid Midi kit (A&A Biotechnology, Poland) was used to obtain the plasmid with the insert.

As a preliminary test to see if the plasmids contained the inserts, gel electrophoresis was performed using the plasmids with the insert and the plasmid without the insert for comparison. If the plasmid, after cloning, was bigger than the plasmid without the insert then it means that, probably, the cloning process was successful.

Sequencing was carried out by Macrogen (The Netherlands). The analysis of sequences was performed on the basic local alignment search tool, BLAST (National Centre for Biotechnology Information).

2.4. Quantitative Real Time RT-PCR

Quantitative real-time reverse transcription PCR (RT-qPCR) has become the method of choice for gene expression analysis. It is a fast and convenient PCR method that uses the phenomenon of fluorescence resonance energy transfer using a DNA-binding dye. The detection of changes in fluorescence intensity during the reaction enables the user to follow the PCR reaction in real time. RT-qPCR comprises several steps: RNA is isolated from target tissue/cells; mRNA is reverse-transcribed to cDNA; gene-specific PCR primers are used to amplify a segment of the cDNA of interest, following the reaction in real time; and the initial concentration of the selected transcript in a specific tissue or cell type is calculated from the exponential phase of the reaction. Relative quantification or absolute quantification can be performed (136).

This technique has revolutionized the field of molecular diagnostics by enabling the shift toward a high-throughput, automated technology with lower turnaround times. By measuring the amount of cellular RNA, it is possible to determine to what extent that gene is being expressed. Expression levels can change dramatically from gene to gene, cell to cell or during various experimental conditions. The quantitative endpoint for real-time PCR is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. By presenting data as the C_T, one ensures that the PCR is in the exponential phase of amplification. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction (the lower the C_T, the greater the amount of amplicon) (137).

The expression level analysis was performed for 4 different genes associated with the cell wall, *CHS1*, *PST1*, *CWP1* and *CWP2*, and that have been verified to be upregulated when there is inactivation of the *FKS1* gene in *S. cerevisiae*. The goal was to check if, besides the possibility of resistant isolates presenting point mutations in the *FKS* gene, other possible alterations in the same gene could lead to the upregulation of the cell wall genes in *Candida* spp. The expression level was verified for 8 isolates (4 *C. albicans*, 3 *C. palmioleophila* and 1 *C. krusei*).

2.4.1. Inoculation and incubation

The isolates were grown on Sabouraud agar plates for 18 – 20 hours at 30°C. Small amounts of biomass from single colonies of each tested strain were suspended in a volume of 4 mL of Sabouraud broth and incubated with continuous shaking for about 5 h at 30°C to achieve OD_{660}=0.6.

2.4.2. RNA isolation

The yeast cells were harvested by centrifugation and mRNA was isolated using the Total RNA Mini Plus Concentrator kit (A&A Biotechnology, Poland) with the acid phenol method (manufacturer's protocol). The isolated RNA was purified with DNase. The reaction mixture composed of 10 µl of sterile RNase-free water, 7 µl of isolated RNA solution, 2 µl of 10 X reaction buffer and 1 µl of DNase (10 U/µl) (A&A Biotechnology, Poland). Incubation followed at 30°C for 30 min and after the mixture was purified with the Total RNA Mini Plus Concentrator kit (A&A Biotechnology, Poland). It was necessary to check the concentration of the mixture to assure that there was enough RNA to proceed with the reverse transcription. NanoDrop1000 (Thermo Scientific, USA) was used for measuring the concentration. The result was 1µg of RNA. Double purification can sometimes result in reduction of efficiency, but it was necessary to obtain a completely pure RNA solution, not contaminated with DNA.

2.4.3. Reverse transcription of mRNA to cDNA

Immediately after purifying mRNA the reverse transcription reaction was carried out using TranScriba kit (A&A Biotechnology, Poland) for the synthesis of the first strand of cDNA from the mRNA template. The reaction was done in two steps. In the first one, the RNA was incubated with 1 µl of oligo(dT)18 solution (100 µM) and RNase-free water added up to volume of 10 µL at 65°C for 5 min to denature the mRNA template secondary structures. After adding the remaining reagents: 4 µL of 5 X reaction buffer, 0.5 µL of RNAse inhibitor, 2 µL of dNTPs Mix solution (2.5 mM of each) and 4 µl of TranScriba Reverse Transcriptase (20 U/µl), the final mixture was incubated at 42°C for 60 min. The reaction was terminated by heating the sample at 70°C for 5 min. The first cDNA strand was stored at -70°C.

2.4.4. Real time PCR

Quantitative analysis of expression of the *CHS1*, *PST1*, *CWP1*, *CWP2* and reference *ACT1* genes was performed by real-time PCR with a LightCycler Nano PCR Real-Time System (Roche, Switzerland). The LightCycler induces fluorescence excitation by a blue light-emitting diode that is read by three silicon photodiodes with different-wavelength filters, allowing detection of spectrally distinct fluorophores. The dye used was SYBR Green 1. This is a nonsequence-specific fluorogenic minor groove DNA-binding dye that intercalates into DNA and it exhibits little fluorescence when unbound in solution but emits a strong fluorescent signal upon binding to DNA. An increase in the fluorescence signal occurs during polymerization and it decreases when DNA is denatured. Real time PCR was performed for a mixture of 10 µl of RealTime 2 X HS-PCR Master Mix Probe (Taq DNA polymerase 0.1 U/µl , 2 X reaction buffer, $MgCl_2$ 10 mM, dNTPs 0.5 mM of each, A&A Biotechnology, Poland), 1 µL of each primer solution (10

µM), 0.5 µl of probe solution (10 µM), 1 µL of total cDNA sample, and distilled water up to the final volume of 20 µl. The PCR reaction stages and conditions are shown in Table 3.

Table 3 – Stages of real time RT-qPCR and respective temperature (°C), hold (s), change in temperature from one PCR step to another over time (ramp, °C/s) and number of cycles.

Stage	Temperature (°C)	Hold (s)	Ramp (°C/s)	Cycles
Hold	95	300	5	-
3-Step Amplification	95	10	5	35
	56	15	4	
	72	15	5	
Hold	72	300	5	
Melting	60	20	4	-
	95	20	0.1	
Hold	40	600	5	

2.4.5. Relative quantification: Livak's method

Quantitative analysis of the relative level of expression of the investigated genes was carried out by using the $2^{-\Delta\Delta C_T}$ method, also called the Livak's method (138). The method allows determination of the relative differences in the expression level of analysed target genes and a reference gene. In order to normalize the results, the expression level of a calibrator is used. For this research, the *C. albicans* 2023 strain isolated at Wrocław Medical University was selected as a calibrator.

The level of expression of target genes in each strain was determined based on comparison of C_T values of amplification of gene of interest and the reference gene (internal control). As a reference gene for this research *ACT1* gene was chosen, a housekeeping gene as its expression is assumed to remain unchanged over a wide range of conditions.

Advantages of the comparative C_T method include ease of use and the ability to present data as fold change in expression (Equation 3). Disadvantages of the comparative C_T method include the assumptions of PCR efficiency must hold or the PCR must be further optimized.

$$\text{Fold change}=2^{-\Delta\Delta C_T} \quad (3)$$

Equation 6 shows the final form of the $2^{-\Delta\Delta C_T}$ equation (138). This form of the equation may be used to compare the gene expression in two different samples. To reach Equation 6 it is needed to have the expression of the gene of interest in a given yeast (C_T gene of interest), expression of the same gene but in the strain used as calibrator (C_T calibrator) and the expression of an internal control gene (C_T reference gene).

$$\Delta C_T \text{ reference} = C_T \text{ gene of interest} - C_T \text{ reference gene} \quad (4)$$

$$\Delta C_T \text{ calibrator} = C_T \text{ gene of interest} - C_T \text{ calibrator} \quad (5)$$

$$2^{-\Delta\Delta C_T} = \Delta C_T \text{ reference} - \Delta C_T \text{ calibrator} \quad (6)$$

$$R = 2^{-\Delta\Delta C_T} \quad (7)$$

$$\text{Expression level} = \log_2(R) \quad (8)$$

The Livak's equation value of parameter R = 1 indicates that the level of the target gene expression in the investigated sample (strain) and internal control gene are the same. A value greater than 1 indicates a higher level of expression of the tested gene in the cells of the investigated strain in comparison to the cells of the calibrator, whereas a significant increase in the level of the gene expression is considered to have occurred when the value of the parameter R is higher than 2.

3. Results and Discussion

3.1. Susceptibility of *Candida* spp.

Categorization of MICs of various antibiotics in AFST depends on breakpoints set by various international agencies. One of the most prevalent guidelines used worldwide is from CLSI whose interpretive cut offs for antifungals is based on MIC distributions, pharmacokinetic–pharmacodynamic properties and the mechanisms of resistance (139).

The results presented are a subset of a larger collection. The detailed procedure, results and discussion of the complete collection is described by the work of Martyna Mroczyńska and Anna Brillowska-Dąbrowska (140).

Table 4 – CLSI breakpoints for *C. albicans*, *C. krusei* and *C. glabrata* regarding echinocandins: anidulafungin (AND), caspofungin (CSP) and micafungin (MCF) *(141)*.

Species	AND	CSP	MCF
C. albicans	≤0.25/≥1	≤0.25/≥1	≤0.25/≥1
C. krusei	≤0.25/≥1	≤0.25/≥1	≤0.25/≥1
C. glabrata	≤0.12/≥0.5	≤0.12/≥0.5	≤0.06/≥0.25

All isolates of *C. albicans* analysed were considered susceptible to all echinocandins and in generally presented low MIC values, far from the breaking points established for this species (Table 4). This is in agreement with the epidemiology reported in Poland, as mentioned in section 1.4. Nevertheless, resistance to echinocandins has been reported among *C. albicans* isolates in other studies but it remains relatively low (1-3%) (142) (143).

There are reports of low susceptibility of *C. krusei* to echinocandins (144) (31) and mutations in resistant isolates of *C. krusei* were reported in the HS1 region of the *FKS1* gene (145). So, the fact that the tested strain 102 was classified as resistant was not completely surprising. Still, reports of resistance to echinocandins from this species are rare, especially in Poland.

C. glabrata 468 was classified as resistant. This species has also been reported as echinocandin resistant and its resistance mechanisms are related with mutations in the *FKS1* and *FKS2* genes. Contrary to *C. albicans* and other *Candida* spp., in *C. glabrata* resistance to echinocandins is more severe, common and often presents as multidrug resistance (146) (147). While echinocandin resistance among *C. glabrata* isolates ranges from 3–5% in population based studies (53), some centres report rates of 10–15% (148) (149).

Table 5 – Minimum inhibitory concentration (MIC) distributions for anidulafungin (AND), caspofungin (CSP) and micafungin (MCF) for 21 *C. albicans*, 2 *C. glabrata*, 1 *C. insconspicua*, 1 *C. krusei* and 5 *C. palmioleophila* isolates. The CLSI breakpoint classification is attributed to each respective MIC and isolate (S: Susceptible; R: Resistant).

Species	Strain	Place of isolation	MIC value (mg/L)			CLSI		
			AND	CSP	MCF	AND	CSP	MCF
C. albicans	26	Wrocław	0.125	0.125	0.063	S	S	S
C. albicans	40	CZD	0.031	0.063	0.016	S	S	S
C. albicans	49	Wrocław	0.125	0.125	0.063	S	S	S
C. albicans	114	Wrocław	0.063	0.063	0.063	S	S	S
C. albicans	125	CZD	0.031	0.016	0.016	S	S	S
C. albicans	185	CZD	0.063	0.016	0.063	S	S	S
C. albicans	266	CZD	0.008	0.016	0.016	S	S	S
C. albicans	286	Gdansk	0.063	0.250	0.031	S	S	S
C. albicans	299	CZD	0.008	0.008	0.016	S	S	S
C. albicans	374	Gdansk	0.008	0.031	0.008	S	S	S
C. albicans	378	Gdansk	0.008	0.031	0.008	S	S	S
C. albicans	380	Gdansk	0.008	0.031	0.008	S	S	S
C. albicans	387	Gdansk	0.008	0.008	0.008	S	S	S
C. albicans	389	Szczecin	0.008	0.031	0.008	S	S	S
C. albicans	395	Szczecin	0.008	0.031	0.008	S	S	S
C. albicans	1010	Wrocław	0.008	0.031	0.016	S	S	S
C. albicans	1296	Wrocław	0.008	0.016	0.016	S	S	S
C. albicans	1768	Wrocław	0.008	0.031	0.016	S	S	S
C. albicans	2023	Wrocław	0.008	0.016	0.016	S	S	S
C. albicans	2029	Wrocław	0.008	0.031	0.016	S	S	S
C. albicans	2608	Wrocław	0.008	0.031	0.008	S	S	S
C. glabrata	373	CZD	0.031	0.063	0.016	S	S	S
C. glabrata	468	Gdansk	0.500	0.500	1.000	R	R	R
C. inconspicua	1444	Wrocław	4.000	4.000	4.000	-	-	-
C. krusei	102	Wrocław	4.000	4.000	4.000	R	R	R
C. palmioleophila	4	Wrocław	4.000	4.000	4.000	-	-	-
C. palmioleophila	368	Szczecin	0.500	1.000	1.000	-	-	-
C. palmioleophila	370	Gdansk	0.008	0.016	0.008	-	-	-
C. palmioleophila	377	Gdansk	0.008	0.016	0.008	-	-	-
C. palmioleophila	405	Szczecin	0.500	4.000	0.500	-	-	-

There are no echinocandin breakpoints established for *C. palmioleophila* and *C. inconspicua* due to the low frequency of occurrence. The high MIC value of 4 mg/L was observed for one of the isolates of *C. palmioleophila*, strain 4. Two strains, 368 and 370, had MIC values ≤ 0.016 mg/L. The other two isolates had a different MIC value depending on the examined echinocandin. Strain 405 presented a very high MIC value for CSP of 4mg/L. A 2011 study that analysed the susceptibility profile of *C. palmioleophila* indicated that the clinical isolates were highly susceptible to echinocandins (AND and MCF with MICs of ≤0.03 mg/L) (36). *C. inconspicua* has been described as susceptible to echinocandins with values between 0.002 – 0.25 mg/L (45). The identified isolate of *C. inconspicua*, for all three echinocandins, had a MIC value of 4 mg/L, which is much higher. The emergence of strains with much higher MIC values than what has been previously recorded might mean that these *Candida* spp. are developing resistant mechanisms against echinocandins.

These results have shown that echinocandin resistance of *Candida* isolates is an ongoing problem, especially in Poland and within non-*albicans* species given the low echinocandin susceptibility for isolates of *C. palmioleophila*, *C. inconspicua, C. krusei* and *C. glabrata.*

3.2. Identification PCR patterns and sequencing of discriminatory bands

3.2.1. Patterns obtained for tested *Candida* spp.

Three primers were tested to see which one would provide results that showed a clear difference between the patterns produced by *C. albicans* and non-*albicans* species. The primers tested (Table 1) were primer A (Figure 7), B (Figure 8) and C (Figures 9 to 14).

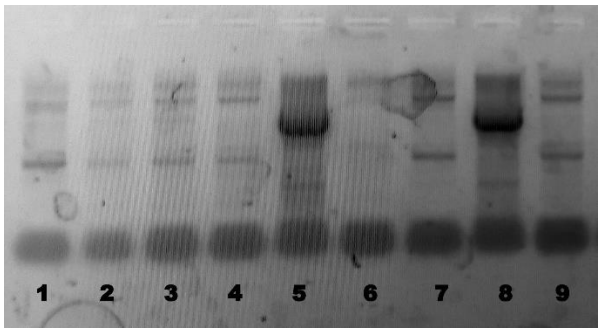


Figure 7 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer A. Lanes: *1* – *C. palmioleophila* 4; 2 – *C.inconspicua* 1444; 3 – *C. albicans* 1296; 4 – *C. palmioleophila* 405; 5 – *C. palmioleophila* 377; 6 – *C. palmioleophila* 368; 7 – *C. krusei* 102; 8 – *C. albicans* 299; 9 – *C. albicans* 286.

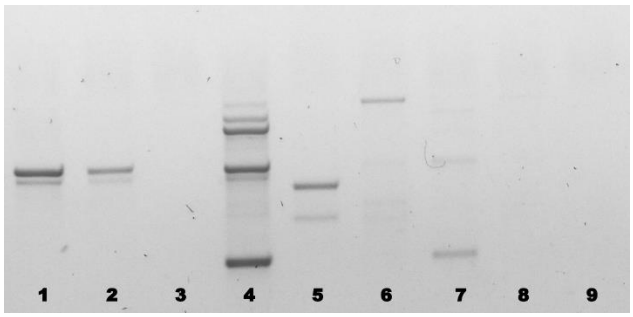


Figure 8 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer B. Lanes: 1 – *C. palmioleophila* 4; 2 – *C. inconspicua* 1444; 3 – *C. albicans* 1296; 4 – *C. palmioleophila* 405; 5 – *C. palmioleophila* 377; 6 – *C. palmioleophila* 368; 7 – *C. krusei* 102; 8 – *C. albicans* 299; 9 – *C. albicans* 286.

Primer C, encoding for the *FKS* gene, was the one that revealed the most range, since results were obtained for a large portion of the collection, and provided the most identifiable differences between *C. albicans*, *C. palmioleophila, C. inconspicua* and *C. krusei*.

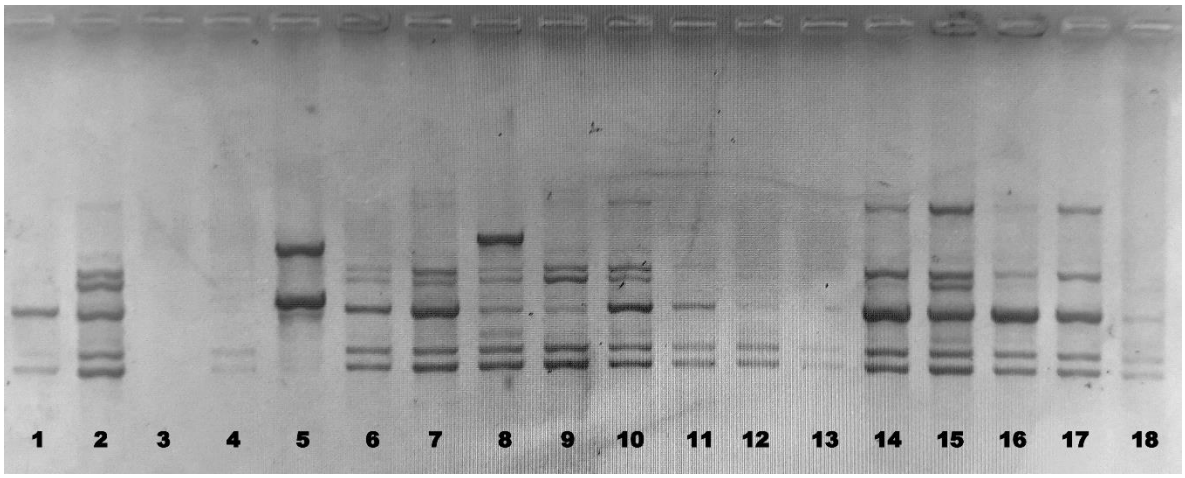


Figure 9 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer C. Lanes: 1 – *C. albicans* 40; 2 – *C. albicans* 72; 3 – *C. glabrata* 127; 4 – *C. parapsilosis* 101; 5 – *C. glabrata* 2235; 6 – *C. albicans* 142; 7 – *C. albicans* 26; 8 – *C. albicans* 79; 9 – *C. albicans* 49; 10 – *C. albicans* 117; 11 – *C. albicans* 51; 12 – *C. albicans* 444 ; 13 – *C. albicans* 17; 14 – *C. albicans* 16; 15 – *C. albicans* 34; 16 – *C. albicans* 33; 17 – *C. albicans* 24; 18 – *C. albicans* 38.

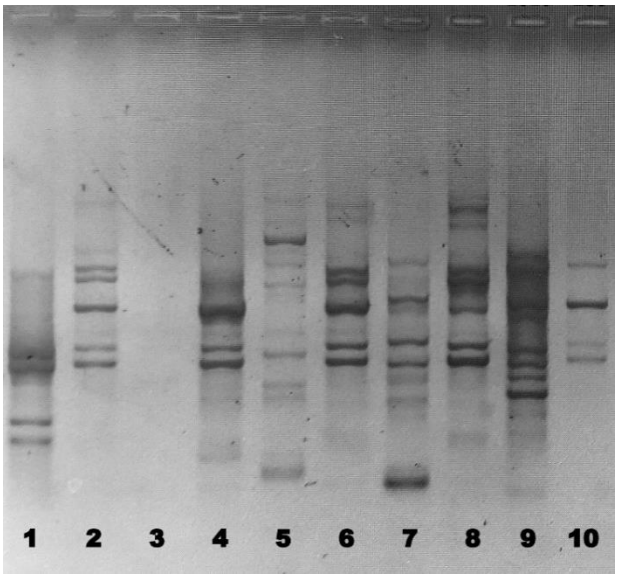


Figure 10 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer C. Lanes: 1 – *C. albicans* 114; 2 – *C. inconspicua* 1444; 3 – *C. palmioleophila* 377; 4 – *C. albicans* 2208; 5 – *C. krusei* 9; 6 – *C. palmioleophila* 4; 7 – *C. albicans* 266; 8 – *C. palmioleophila* 368; 9 – *C. albicans* 2029; 10 – *C. albicans* 286.

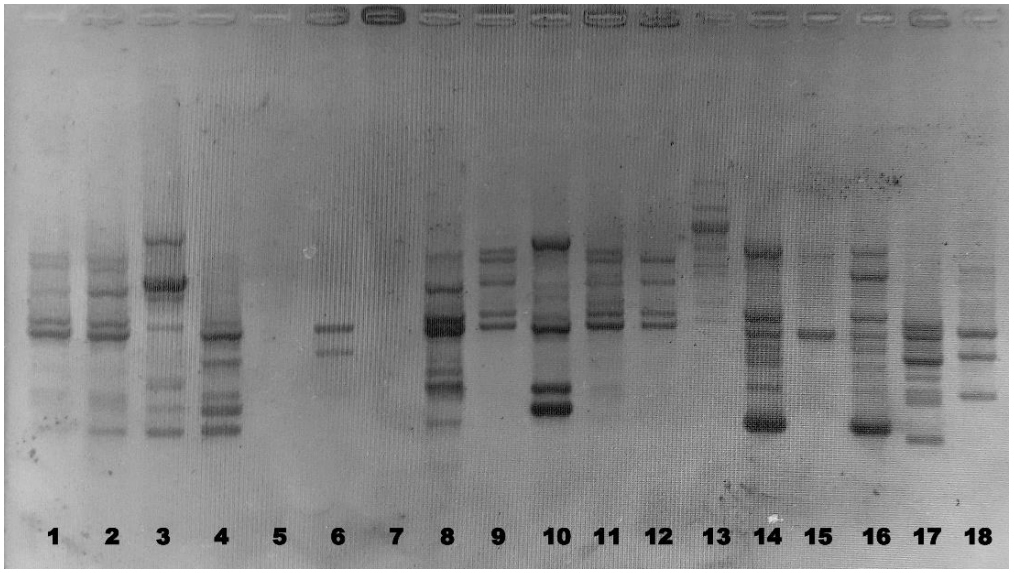


Figure 11 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer C. Lanes: 1 – *C. albicans* 472; 2 – *C. albicans* 2048; 3 – *C. glabrata* 1104; 4 – *C. albicans* 185; 5 – *C. glabrata* 82; 6 – *C. glabrata* 118; 7 – *C. glabrata* 81; 8 – *C. albicans* 109; 9 – *C. albicans* 2023; 10 – *C. albicans* 99; 11 – *C. albicans* 561; 12 – *C. albicans* 52; 13 – *C. krusei* 268; 14 – *C. albicans* 388; 15 – *C. albicans* 2204; 16 – *C. parapsilosis* 441; 17 – *C. glabrata* 468; 18 – *C. glabrata* 1150.

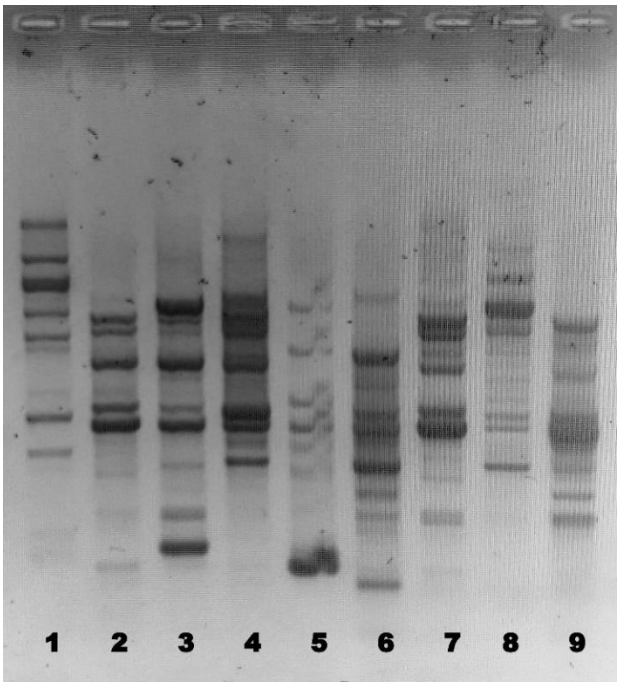


Figure 12 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer C. Lanes: 1 – *C. krusei* 9; 2 – *C. palmioleophila* 4; 3 – *C. albicans* 1296; 4 – *C. palmioleophila* 405; 5 – *C. albicans* 1027; 6 – *C. albicans* 1010; 7 – *C. palmioleophila* 368; 8 – *C. krusei* 102; 9 – *C. albicans* 125.

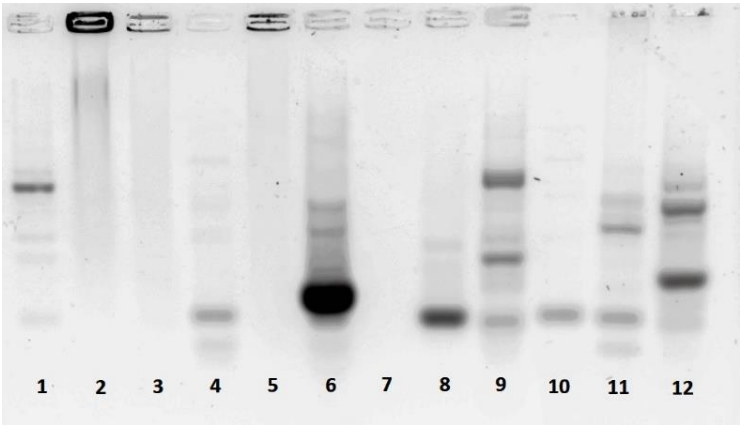


Figure 13 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer C. Lanes: 1 – *C. glabrata* 260; 2 – *C. glabrata* 273; 3 – *C. glabrata* 513; 4 – *C. albicans* 299; 5 – *C. glabrata* 316; 6 – *C. glabrata* 365; 7 – *C. albicans* 366; 8 – *C. palmioleophila* 370; 9 – *C. glabrata* 373; 10 – *C. palmioleophila* 377; 11 – *C. parapsilosis* 381; 12 – *C. albicans* 387.

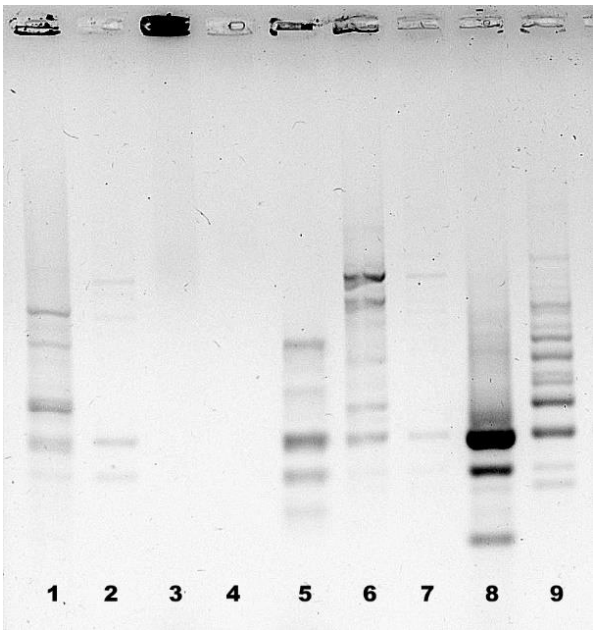


Figure 14 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer C. Lanes: 1 – *C. glabrata* 276; 2 – *C. glabrata* 240; 3 – *C. albicans* 89; 4 – *C. albicans* 71; 5 – *C. albicans* 50; 6 – *C. albicans* 395; 7 – *C. glabrata* 2181; 8 – *C. glabrata* 31; 9 – *C. albicans* 391.

Primer C was designed for the *FKS* gene, a gene that is directly related to echinocandin resistance for *Candida* spp. It is possible that strains with low susceptibility to echinocandins have point mutations on this gene (as described in section 1.3.3). This would be consistent with the fact that the patterns would vary according the existence of these mutations. The MIC values can provide further information if point mutations on the *FKS* gene can be identified through PCR and predict susceptibility.

Unpublished MIC information was used only for consulting and was not included in the previous section 3.1 because they are part of a previously mentioned study by Martyna Mroczyńska and Anna Brillowska-Dąbrowska (2019) (140) (Appendix).

C. palmioleophila strains presented a few differences between them. It was possible to obtain discernible patterns for strains 4, 368 and 405. This was not the case for strains 370 and 377. There is a clear difference between these two sets, and it could mean that there is a difference between the *FKS* genes of the strains of the two sets. Given the MIC information, strains 370 and 377 have the lowest MIC values of all *C. palmioleophila* isolates, much lower than strain 4, 368 and 405 (Table 5).

Regarding *C. albicans* patterns, the most common and distinguishable pattern is shown for 20 strains. Other patterns associated with this species included: many bands (11 strains) and only one band (3 strains). Other patterns/no pattern at all were also verified (6 strains). Table 6 displays the different *C. albicans* strains and correspondent patterns. When compared to the results from section 3.1 there was no clear relation between susceptibility and patterns. When it comes to the common band pattern, MICs range between 0.008 – 0.25 mg/L for AND, 0.016 –

0.25 mg/L for CSP and 0.016 – 0.063 mg/L for MCF. For the many bands pattern, MICs range between 0.008 – 1 mg/L for AND, 0.016 – 4 mg/L for CSP and 0.008 – 1 mg/L for MCF. For the only one band pattern, MICs range between 0.008 – 0.031 mg/L for AND, 0.008 – 0.250 mg/L for CSP and are all 0.016 mg/L for MCF. Finally, for other patterns, MICs range between 0.008 – 0.063 mg/L for AND, 0.008 – 0.125 mg/L for CSP and 0.008 – 0.063 mg/L for MCF. The ranges presented include unpublished results. It is interesting to notice that the only two resistant strains from this species, 388 and 391 (unpublished results), both presented a lot more bands than the common pattern.

Table 6 – Types of patterns obtained for different strains of *C. albicans* following PCR fingerprinting with primer C.

Species	Pattern	Strains
C. albicans	Common	16, 17, 24, 26, 33, 34, 38, 49, 51, 52, 72, 79, 117, 142, 286, 444, 472, 561, 2023, 2048
	Many bands	50, 99, 109, 125, 185, 388, 391, 395, 1010, 1027, 1296, 2029
	One band	40, 299, 2204
	Others	71, 89, 114, 366, 387, 2208

Regarding *C. krusei*, patterns for strain 9 and 268 are similar but strain 102 presents some identifiable differences. Strains 9 and 268 have a pattern with individual and spaced bands while strain 102 displays more bands and more compressed. These differences may relate to susceptibility given that strain 102 is resistant to all three echinocandins (Table 5) and strains 9 and 268 are resistant to only CSP (unpublished results).

For *C. inconspicua* 1444 the result of genotyping did not present a distinctive characteristic pattern when compared to *C. albicans* strains, in fact the pattern presented is similar to *C. albicans* common pattern. This can have implications such as, the strain has been misidentified as *C. inconspicua* or *C. albicans* and *C. inconspicua* are phylogenetically close. It is not possible though to take conclusions from this given that only one strain was analysed. Also *C. glabrata* and *C. parapsilosis,* unfortunately, did not display a common pattern or any perceivable relation between the MIC values of the strains and the obtained patterns. This does not mean that there can't be a relation between them, to verify it a bigger sample would be needed. There were 18 *C. glabrata* and 3 *C. parapsilosis* isolates tested, which not a representative sample of these species.

The PCR profiles obtained were informative and generated clear and distinct patterns for most of the *Candida* spp. Major discriminatory bands were identified for *C. palmioleophila* and *C.krusei*.

Some of the isolates did not show any PCR product at all. This could be connected to reasons like an unsuccessful DNA isolation, contamination of the colonies, contamination of the PCR mixture/product, or the amount of DNA sample/primer used.

3.2.2. Discriminatory band sequencing

After visual analysis the of gels it was decided to proceed with sequencing of the bands marked in Figure 15. Two bands from *C. palmioleophila* 4 and 368 and *C. krusei* 9 and 102 were chosen to proceed with cloning and sequencing. For *C. inconspicua* 1444 one band was also selected, even given the fact that its pattern did didn't seem to be any different than the *C. albicans* most common pattern. It was, although, decided, since this is a rare yeast, to sequence a very discernible band that was displayed in order to try to get possible novel information.

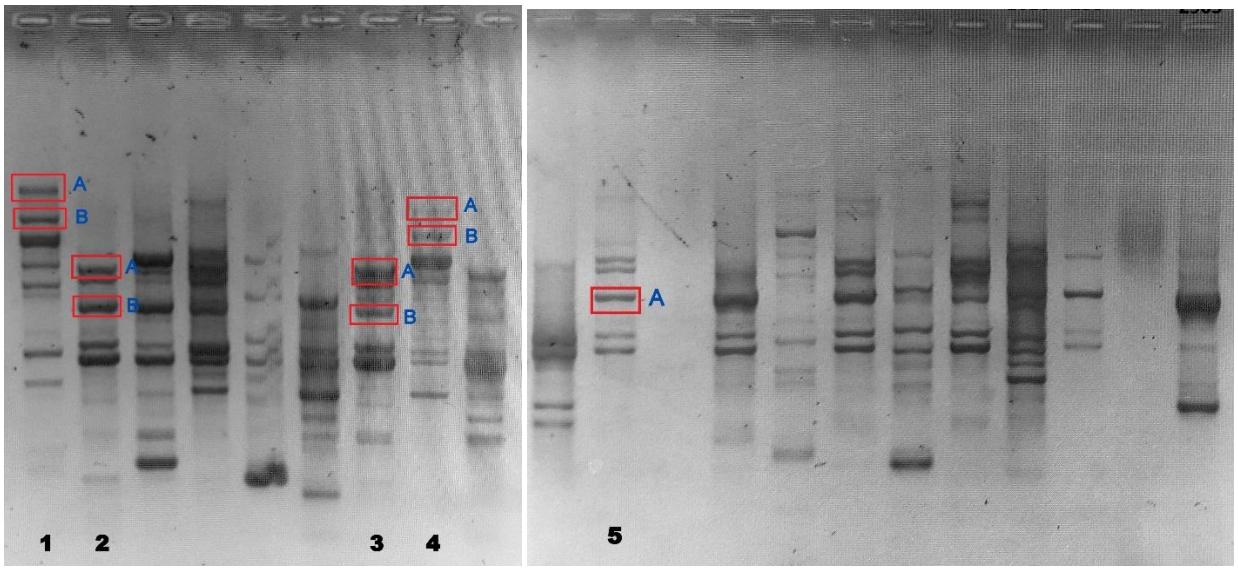


Figure 15 – Boxed bands in red represent the genes of interest chosen that were used in the cloning process from Figures 10 and 12. Lanes: 1 – *C. krusei* 9 and interest bands A and B; 2 – *C. palmioleophila* 4 and interest bands A and B; 3 – *C. palmioleophila* 368 and interest bands A and B; 4 – *C. krusei* 102 and interest bands A and B; 5 – *C. inconspicua* 1444 and interest band A.

The blue/white colony screening revealed white colonies for all the selected bands. Before sending the final product for sequencing a preliminary test was made. Using the plasmids with the insert and the plasmid without the insert electrophoresis was performed. The goal of this test was to determinate if, when compared to the plasmid without the insert, the plasmids with the insert were bigger, meaning they probably contained the gene of interest.

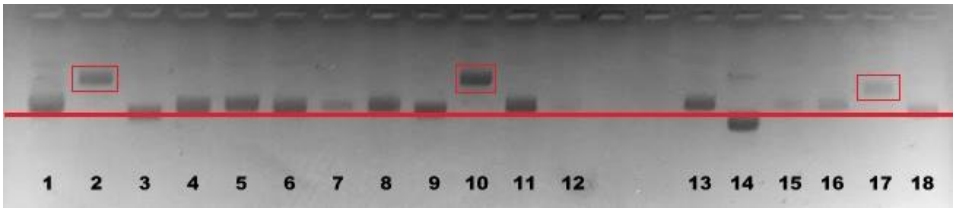


Figure 16 – Agarose gel electrophoresis (1.5% agarose) of plasmid products from cloning. Lanes: 1 – Plasmid without insert; 2 to 6 – Plasmid with *C. palmioleophila* 4 insert for band A; 7 to 9 – Plasmid with *C. palmioleophila* 4 insert for band B; 10 and 11 – Plasmid with *C. krusei* 9 insert for band A; 12 to 16 – Plasmid with *C. krusei* 102 insert for band A; 17 and 18 –Plasmid

with *C. palmioleophila* 368 insert for band B; The red line marks the weight of the plasmid without the insert. The plasmids with insert with weight superior to the plasmid without the insert were boxed in red.

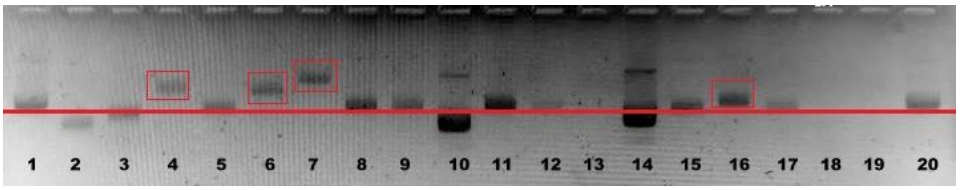


Figure 17 – Agarose gel electrophoresis (1.5% agarose) of plasmid products from cloning. Lanes: 1 – Plasmid without insert; 2 to 3 – Plasmid with *C. palmioleophila* 368 insert for band A; 4 to 7 – Plasmid with *C. palmioleophila* 368 insert for band B; 8 to 12 – Plasmid with *C. krusei* 102 insert for band B; 13 to 19 – Plasmid with *C. inconspicua* 1444 insert for band A; 20 –Plasmid without insert; The red line marks the weight of the plasmid without the insert. The plasmids with insert with weight superior to the plasmid without the insert were boxed in red.

From the 38 plasmids obtained by cloning only 7 were sent for sequencing, as displayed in Figures 16 and 17. No plasmid seemed to have acquired the inserts from *C. krusei* 102. The 7 selected plasmids contained the gene of interest from *C. palmioleophila* 4 (A) and 368 (B), *C. krusei* 9 (A) and *C. inconspicua* 1444 (A). Unfortunately, the sequencing results did not identify any novel or known genes because most of the sequencing results did not have discernible sequences or the sequences belonged to the plasmid, revealing that cloning was not successful. Several factors may have affected the efficiency of the cloning process. At the PCR level, since the gene inserts were unknown, they might have contained repeated sequences resulting in a mixture of many different amplicons which lead to improper annealing during successive rounds of PCR (150). Another limitation might have been that the primers used for PCR could have annealed non-specifically to sequences that were similar, but not completely identical to target DNA. In addition, incorrect nucleotides could have been incorporated into the PCR sequence by the DNA polymerase, although at a very low rate. Because PCR is a highly sensitive technique, any form of contamination of the sample by even trace amounts of DNA could have produced misleading results (151). Exposure of DNA to UV radiation in standard preparative agarose gel electrophoresis procedure for as little as 1 min can damage the DNA, and this could have significantly reduced the transformation efficiency (152). The method of preparation of competent cells, the length of time of heat shock, temperature of heat shock, incubation time after heat shock, growth medium used, and various additives, all could have affected the transformation efficiency of the cells (153). Plasmid characteristics might have also contributed such as plasmid size and form – a study done in *E. coli* found that transformation efficiency declines linearly with increasing plasmid size, this means that larger plasmids transform less well than smaller plasmids; supercoiled plasmids also have a slightly better transformation efficiency than relaxed plasmids (154). The screening of colonies might have also been an important factor at play. Some white colonies may not have contained the desired recombinant vector for several reasons. The ligated DNA could not have been the correct one, and it is possible for some linearized vector to be transformed, its ends repaired and ligated together such that no lacZα is produced and no blue colonies may be formed. A colony with no vector at all will also appear white and may sometimes appear as satellite colonies after the antibiotic used has been depleted. It is also possible that blue colonies may have contained the insert. This occurs when the insert is in frame

with the lacZα gene and a STOP codon is absent in the insert. This can lead to the expression of a fusion protein that is still functional as lacZα (155).

3.3. Expression level analysis for *CHS1*, *PST1*, *CWP1* and *CWP2* genes

The real time RT-qPCR was performed for genes *CHS1*, *PST1*, *CWP1* and *CWP2* to check their expression levels on 8 *Candida* spp. isolates (4 *C. albicans*, 1 *C. krusei*, 3 *C. palmioleophila*). The obtained C_T values for the reference gene are shown in Table 7. The results for C_T values and respective expression level ($log_2(R)$) by application of Livak's method are displayed for genes *CHS1*, *PST1*, *CWP1* and *CWP2* in Tables 8, 9, 10 and 11 respectively.

Table 7 – *ACT1* gene expression. C_T average values and respective standard deviation for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029.

ACT1	
Species	**C_T average**
***C. albicans* 395**	33.5±0.511
***C. albicans* 1010**	25.0±0.422
***C. albicans* 2023**	26.0±0.357
***C. albicans* 2029**	29.2±0.107
***C. krusei* 102**	34.7±0.893
***C. palmioleophila* 4**	31.6±1.60
***C. palmioleophila* 368**	16.9±0.184
***C. palmioleophila* 370**	21.0±0.586

Table 8 – *CHS1* gene expression using *C. albicans* 2023 as a calibrator. Livak's method parameters: C_T average values and respective standard deviation; ΔC_T reference values; ΔC_T calibrator values; $\Delta\Delta C_T$ values; R values; $log_2(R)$ values that translate in expression level, for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029.

CHS1						
Species	**C_T average**	**ΔC_T reference**	**ΔC_T calibrator**	**$\Delta\Delta C_T$**	**R**	**$log_2(R)$**
***C. albicans* 395**	26.4±0.537	-7.09	-3.56	-3.53	11.6	3.53
***C. albicans* 1010**	27.8±2.25	2.78	-2.15	4.93	3.29E-02	-4.93
***C. albicans* 2023**	29.9±0.512	3.92	0.00	3.92	6.61E-02	-3.92
***C. albicans* 2029**	24.2±0.236	-4.98	-5.73	0.753	0.593	-0.753
***C. krusei* 102**	27.0±0.485	-7.68	-2.94	-4.74	26.7	4.74
***C. palmioleophila* 4**	32.5±0.804	0.893	2.58	-1.68	3.21	1.68
***C. palmioleophila* 368**	21.8±0.107	4.87	-8.18	13.1	1.18E-04	-13.1
***C. palmioleophila* 370**	28.4±0.559	7.42	-1.53	8.94	2.03E-03	-8.94

Table 9 – *PST1* gene expression using *C. albicans* 2023 as a calibrator. Livak's method parameters: C_T average values and respective standard deviation; ΔC_T reference values; ΔC_T calibrator values; $\Delta\Delta C_T$ values; R values; $\log_2(R)$ values that translate in expression level, for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029.

PST1						
Species	**C_T average**	**ΔC_T reference**	**ΔC_T calibrator**	**$\Delta\Delta C_T$**	**R**	**$\log_2(R)$**
***C. albicans* 395**	32.5±3.70	-5.55	-2.66	-2.89	7.40	2.89
***C. albicans* 1010**	30.0±0.668	4.98	-0.59	5.57	2.11E-02	-5.57
***C. albicans* 2023**	32.5±1.14	4.56	0.00	4.56	4.23E-02	-4.56
***C. albicans* 2029**	26.3±0.0778	-2.84	-4.24	1.40	0.380	-1.40
***C. krusei* 102**	35.9±0.622	1.24	5.34	-4.10	17.1	4.10
***C. palmioleophila* 4**	34.9±0.746	3.32	4.36	-1.04	2.06	1.04
***C. palmioleophila* 368**	24.3±0.199	7.44	-6.25	13.7	7.55E-05	-13.7
***C. palmioleophila* 370**	27.6±0.587	6.64	-2.95	9.59	1.30E-03	-9.59

Table 10 – *CWP1* gene expression using *C. albicans* 2023 as a calibrator. Livak's method parameters: C_T average values and respective standard deviation; ΔC_T reference values; ΔC_T calibrator values; $\Delta\Delta C_T$ values; R values; $\log_2(R)$ values that translate in expression level, for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029.

CWP1						
Species	**C_T average**	**ΔC_T reference**	**ΔC_T calibrator**	**$\Delta\Delta C_T$**	**R**	**$\log_2(R)$**
***C. albicans* 395**	31.5±0.177	-1.95	8.25	-10.2	1.17E+03	10.2
***C. albicans* 1010**	24.4±0.739	-0.587	1.15	-1.74	3.34	1.74
***C. albicans* 2023**	23.3±0.229	-2.75	0.00	-2.75	6.71	2.75
***C. albicans* 2029**	23.1±0.393	-6.07	-0.160	-5.91	60.3	5.91
***C. krusei* 102**	31.8±1.27	-2.86	8.55	-11.4	2.71E+03	11.4
***C. palmioleophila* 4**	17.5±0.123	-14.2	-5.81	-8.35	3.26E+02	8.35
***C. palmioleophila* 368**	18.2±0.135	1.31	-5.08	6.38	1.20E-02	-6.38
***C. palmioleophila* 370**	19.1±0.136	-1.93	-4.21	2.28	0.206	-2.28

Table 11 - *CWP2* gene expression using *C. albicans* 2023 as a calibrator. Livak's method parameters: C_T average values and respective standard deviation; ΔC_T reference values; ΔC_T calibrator values; $\Delta\Delta C_T$ values; R values; $\log_2(R)$ values that translate in expression level, for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029.

CWP2						
Species	**C_T average**	**ΔC_T reference**	**ΔC_T calibrator**	**$\Delta\Delta C_T$**	**R**	**$\log_2(R)$**
***C. albicans* 395**	28.8±0.760	-4.68	-4.00E-02	-4.64	25.0	4.64
***C. albicans* 1010**	28.2±0.107	3.19	-0.62	3.81	7.11E-02	-3.81
***C. albicans* 2023**	28.8±0.170	2.81	0.00	2.81	0.143	-2.81
***C. albicans* 2029**	26.6±0.564	-2.60	-2.24	-0.360	1.28	0.360
***C. krusei* 102**	29.4±0.629	-5.30	0.558	-5.85	57.8	5.85
***C. palmioleophila* 4**	32.9±0.805	1.32	4.12	-2.80	6.95	2.80
***C. palmioleophila* 368**	19.9±0.0513	3.01	-8.93	11.9	2.55E-04	-11.9
***C. palmioleophila* 370**	28.1±0.328	7.10	-0.727	7.83	4.39E-03	-7.83

To better visualize the results these were displayed using diagrams of the expression level of each gene and respective species and strains (Figures 18 to 21).

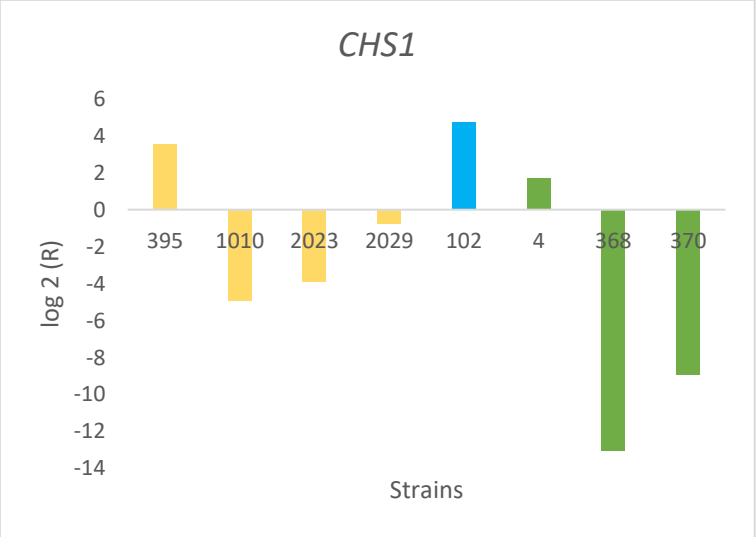


Figure 18 – Expression level of *CHS1* gene regarding 8 isolates: *C. albicans* 395, 1010, 2023 and 2029 (yellow); *C. krusei* 102 (blue) and *C. palmioleophila* 4, 368 and 370 (green), in relation to a calibrator assigned as *C. albicans* 2023.

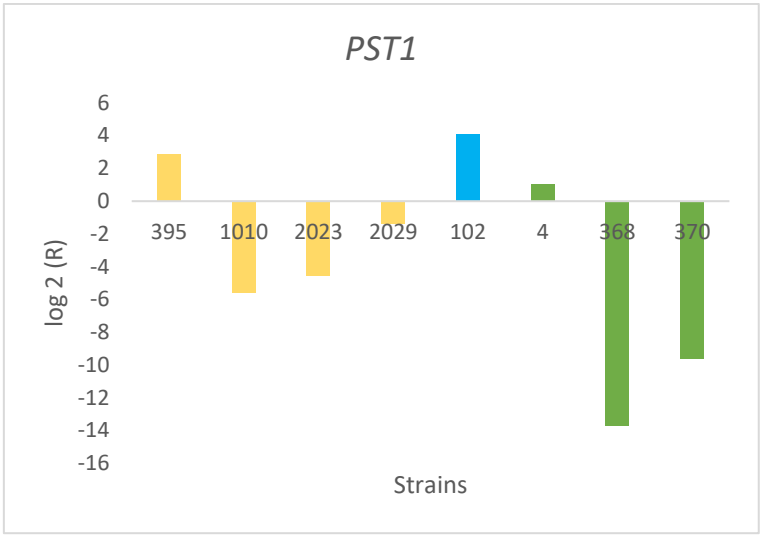


Figure 19 – Expression level of *PST1* gene regarding 8 isolates: *C. albicans* 395, 1010, 2023 and 2029 (yellow); *C. krusei* 102 (blue) and *C. palmioleophila* 4, 368 and 370 (green), in relation to a calibrator assigned as *C. albicans* 2023.

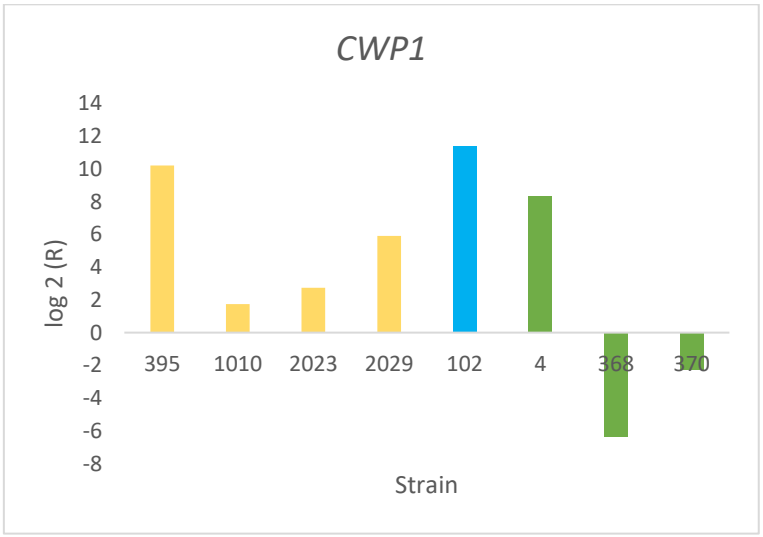


Figure 20 – Expression level of *CWP1* gene regarding 8 isolates: *C. albicans* 395, 1010, 2023 and 2029 (yellow); *C. krusei* 102 (blue) and *C. palmioleophila* 4, 368 and 370 (green), in relation to a calibrator assigned as *C. albicans* 2023.

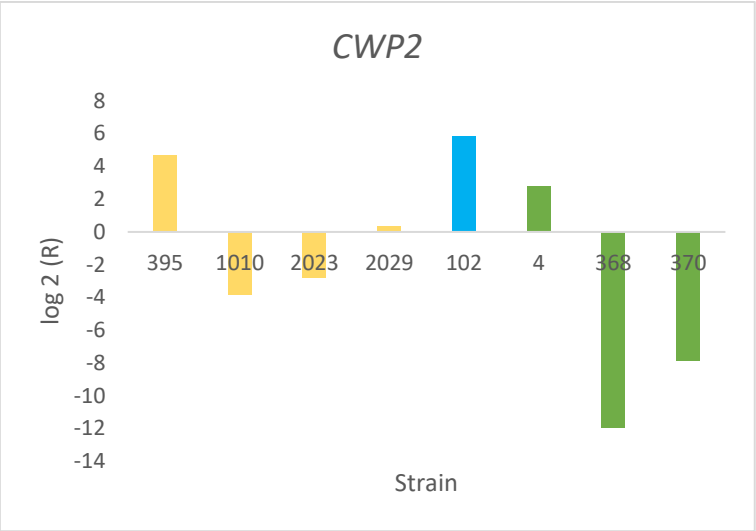
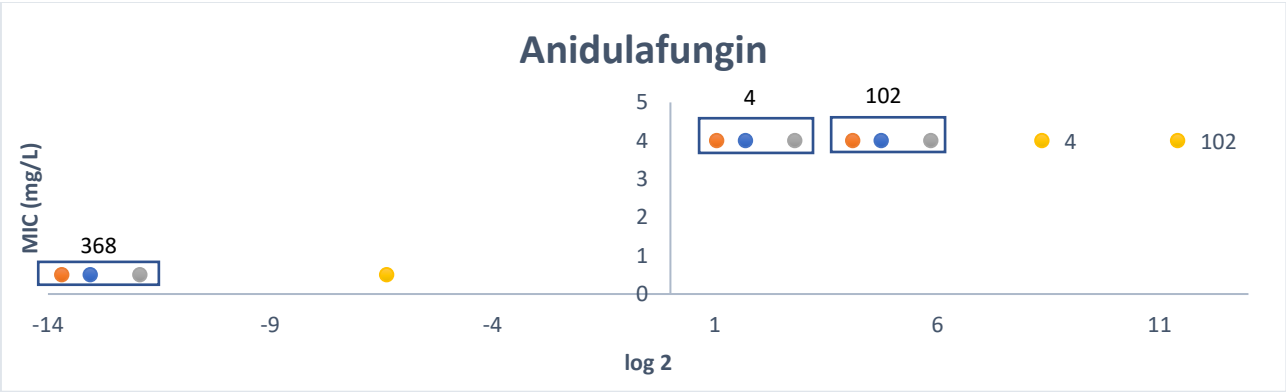


Figure 21 – Expression level of *CWP2* gene regarding 8 isolates: *C. albicans* 395, 1010, 2023 and 2029 (yellow); *C. krusei* 102 (blue) and *C. palmioleophila* 4, 368 and 370 (green), in relation to a calibrator assigned as *C. albicans* 2023.

As stated in section 1.5.1 there is evidence that when there are alterations in the *FKS1* gene, genes *CHS1*, *PST1*, *CWP1* and *CWP2* are upregulated. The goal was to check if this would be the case for the tested isolates. According to the results all genes are upregulated for *C. albicans* 395, *C. krusei* 102 and *C. palmioleophila* 4 and downregulated in *C. palmioleophila* 368 and 370. On the other hand, *CWP1* is also upregulated for *C. albicans* 1010, 2023 and 2029, while *CHS1*, *PST1* and *CWP2* are downregulated.

Other diagrams were used (Figures 22 to 24) to better visualize the relation between MIC values for each echinocandin and respective expression levels for every isolate.



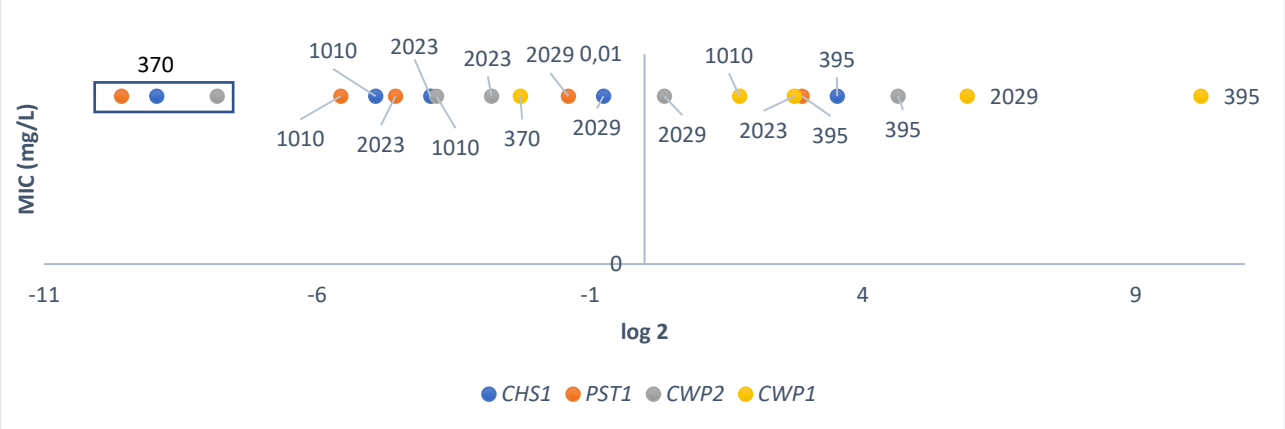
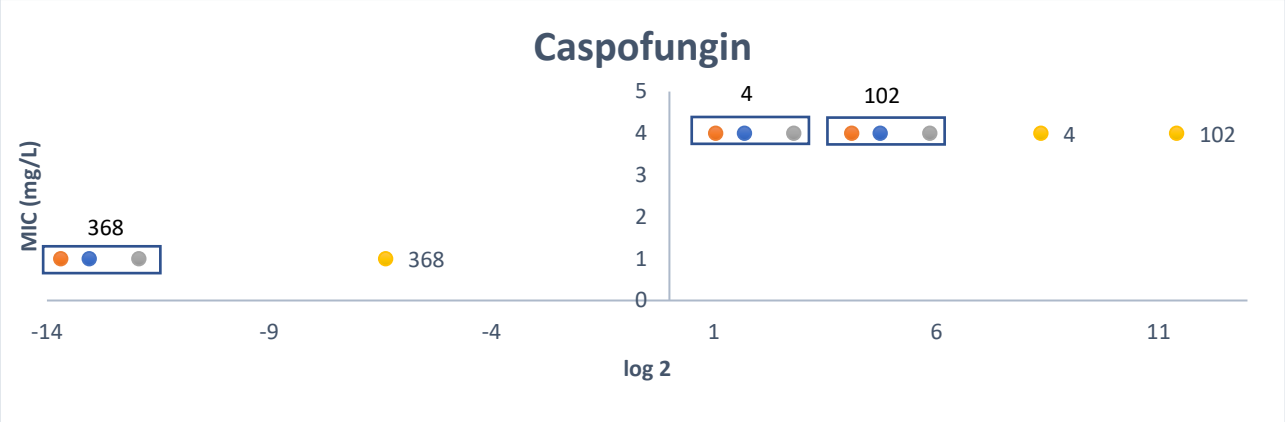


Figure 22 – Plot of relative *CHS1*, *PST1*, *CWP1* and *CWP2* gene expression versus MIC (mg/L) values for AND for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029 isolates. Gene transcript levels were normalized against *ACT1* transcript levels. *C. albicans* 2023 was used as calibrator.



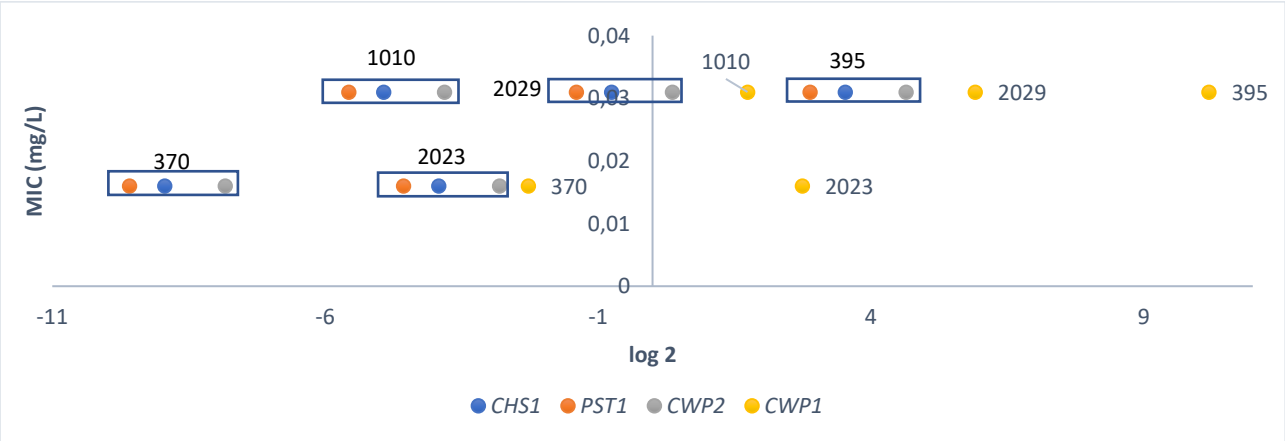


Figure 23 – Plot of relative *CHS1*, *PST1*, *CWP1* and *CWP2* gene expression versus MIC (mg/L) values for CSP for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029 isolates. Gene transcript levels were normalized against *ACT1* transcript levels. *C. albicans* 2023 was used as calibrator.

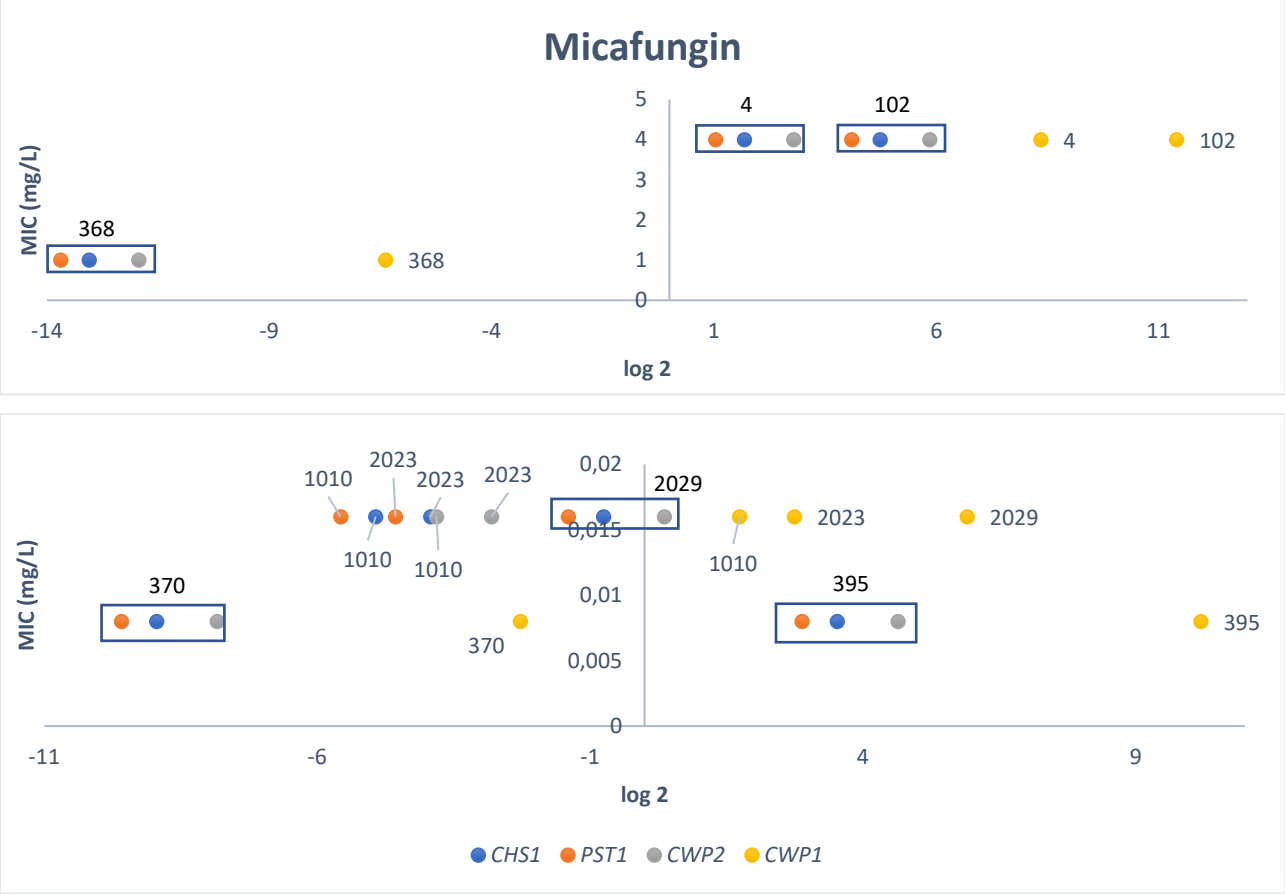


Figure 24 – Plot of relative *CHS1*, *PST1*, *CWP1* and *CWP2* gene expression versus MIC (mg/L) values for MCF for *C. palmioleophila* 4, 368, 370; *C. krusei* 102; and *C. albicans* 395, 1010, 2023, 2029 isolates. Gene transcript levels were normalized against *ACT1* transcript levels. *C. albicans* 2023 was used as calibrator.

C. palmioleophila 4 and *C. krusei* 102 have similar and high expression levels for all genes. It means the genes are upregulated in these isolates. This is an interesting comparison given that the MIC values for these two strains are the same (Table 5). *C. krusei* 102 has been considered resistant and even though that there are no breakpoints for *C. palmioleophila* the correspondent MIC value is quite high. Less susceptible strains possibly have, besides point mutations, the *FKS1* gene altered and therefore the tested genes were upregulated. On the other hand, some evidence that might support the fact that these genes are connected to alterations in *FKS1* and that they are connected to the resistance mechanism in *C. palmioleophila* is that for the other two strains, 368 and 370, their MIC values are low compared to strain 4, meaning that strains 368 and 370 have higher susceptibility to echinocandins. The genes are also downregulated in these strains, contrary to strain 4 where they are upregulated.

C. albicans 1010 and 2023 share resemblance between their MIC values and expression levels. *CHS1*, *PST1* and *CWP2* show low expression levels and *CWP1* high expression levels. For *C. albicans* 2029 though, *CWP2* also shows high expression levels. *C. albicans* 395, has all genes with high expression levels. It seems that there is not a consensus between the *C. albicans* strains and their level of gene expression. The MIC values of the 4 *C. albicans* isolates are the same for AND. For CSP, strain 2023 is the only strain presenting a lower MIC value and for MCF the MIC value for strain 395 is lower than the others. All the strains, as mentioned before, are classified as susceptible to

echinocandins (Table 5). Given this analysis and the fact that the expression levels for strain 395 are much higher than of the other *C. albicans* strains, it seems that there is not an evident connection between the studied genes and *C. albicans* resistance to echinocandins. It could mean though that the *FKS1* gene is altered in this strain, but it does not present the point mutations that confer resistance to echinocandins. However, since there are no representatives of resistant *C. albicans* strains in this study, it is not possible to draw definite conclusions.

4. Conclusions and Perspectives

The results from the AFST showed that frequency of non-*albicans Candida* spp. is increasing and so are their less susceptible strains. The fact that there are still no breakpoints for rare yeasts such as *C. palmioleophila* and *C. inconspicua* does not allow the classification of strains with high MIC levels as resistant. However, the elevated MIC values and previous information from literature suggests that resistance mechanisms have been evolving in these species. *C. krusei* and *C. glabrata* strains were classified as resistant to all three echinocandins. Resistant strains and their mechanisms of resistance for these species have been described before but this is not common for *C. krusei*. In terms of epidemiology in Poland though these results are important and highly informative given that they are the first to describe the emergence of non-*albicans Candida* with low susceptibility to echinocandins in the country.

PCR fingerprinting using the primer encoding the *FKS* gene, is proposed as a simple, reliable and highly reproducible diagnosis tool that allows to visualize and identify the different species of *Candida*. It is also fast and cost-effective. *C. albicans* displayed a unique pattern for most of its strains that allowed for a quick visual identification. There were no evident connections between patterns and susceptibility, within the studied sample of isolates but it is worth noting that resistant strains (unpublished results) seemed to fit in within the many band pattern. In the future, it would be interesting to analyse the patterns and ASFT results of a larger collection containing susceptible and resistant strains. Results were particularly interesting for *C. palmioleophila*. The strains of this species displayed an exclusive pattern with discriminatory bands and the patterns seemed to differ according to the susceptibility of the strains regarding echinocandins. This is important information given that the primer used was designed for the *FKS* gene. Unfortunately for *C. inconspicua* it was not possible to find a distinctive pattern since it displayed a pattern similar to the common pattern of *C. albicans*. Two possibilities are that *C. inconspicua* was misidentified or this species and *C. albicans* are phylogenetically close. The expected result was that the pattern would be more like *C. krusei* given that *C. inconspicua* is more closely related to this species. *C. krusei* strains displayed an exclusive pattern with major discriminatory bands. There seems to be a connection between patterns and susceptibility given that the pattern belonging to the resistant strain had a lot more bands than the other two strains resistant to only CSP (unpublished results). This was however a very small sample of this species. More strains should be added in the future to confirm this connection. *C. glabrata* and *C. parapsilosis,* unfortunately, did not display an identifiable pattern or any correlation with the ASFT. Once again, the sample and susceptibility range for this species was limited, so in future assays it would be necessary to widen these variables to extract more significant results. In this study it was not possible to conclude the genotyping process and demonstrate if PCR fingerprinting data reflects phylogenetic relationships between a given set of isolates because it was not possible to successfully clone the discriminatory bands.

The results from real time RT-qPCR show that the relation between possible alterations on the *FKS1* gene and the upregulation of *CHS1*, *PST1*, *CWP1* and *CWP2* is verified for *C. palmioleophila* 4 and *C. krusei* 102, two isolates with low echinocandin susceptibility – meaning that the *FKS1* gene is probably altered in these isolates, besides possible point mutations that confer echinocandin resistance. Since there are no breakpoints for *C. palmioleophila* there is

also the possibility though that the levels of expression might relate to stress adaptation responses resulting in high MIC values. It can mean that strains have developed drug tolerance which is nothing less than an intermediate stage for development of resistance and just as worrisome. On the other hand, given the upregulation of the genes in *C. albicans* 395 it is possible that the *FKS1* gene is altered in this isolate. *C. albicans* 395 is classified as susceptible but the levels of expression of all genes match the ones for *C. palmioleophila* 4 and *C. krusei* 9, which means that besides the *FKS1* gene being altered in this isolate there are no point mutations that confer echinocandin resistance. It would be necessary to repeat the study and add a resistant strain of *C. albicans* to reach conclusions regarding this species.

The results of these set of experiments have shown that amongst *Candida* spp., there are important connections between cell wall dynamics and resistance to antifungal drugs. It may not be surprising that such a connection exists, given that most antifungal agents target the fungal membrane or cell wall. It has also shown that *Candida* spp. are developing resistance mechanisms that decrease their susceptibility, creating a higher risk for clinical infections. The impact that these strains may have in the clinical setting is an ever-growing concern. This could be associated with poorer clinical outcomes for patients and breakthrough infections during antifungal treatment and prophylaxis, and increased healthcare costs. This is particularly important when it comes to echinocandins that, as previously mentioned, are currently on the front line of antifungal treatment. Echinocandin resistance among *Candida* spp. is uncommon, except with *C. glabrata* where high level resistance is reported (often associated with azole resistance). The rise in resistant strains against these antifungals is worrisome given the limited treatment options for *Candida* infections. It is important to continue to try to understand how the mechanisms of resistance and the cellular responses to maintain cell wall integrity work in order to develop new strategies to fight these infections.

5. References

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6. Appendix

Table 12 – Complete list of isolates tested in section 3.2 and their respective MIC values.

Number	Species and strains	MIC values (mg/L)
1.	*C. albicans* 16;	*Unpublished results*
2.	*C. albicans* 17;	*Unpublished results*
3.	*C. albicans* 24;	*Unpublished results*
4.	*C. albicans* 26;	*Table 6*
5.	*C. albicans* 33;	*Unpublished results*
6.	*C. albicans* 34;	*Unpublished results*
7.	*C. albicans* 38.	*Unpublished results*
8.	*C. albicans* 40;	*Table 6*
9.	*C. albicans* 49;	*Table 6*
10.	*C. albicans* 50;	*Unpublished results*
11.	*C. albicans* 51;	*Unpublished results*
12.	*C. albicans* 52;	*Unpublished results*
13.	*C. albicans* 71;	*Unpublished results*
14.	*C. albicans* 72;	*Unpublished results*
15.	*C. albicans* 79;	*Unpublished results*
16.	*C. albicans* 89;	*Unpublished results*
17.	*C. albicans* 99;	*Unpublished results*
18.	*C. albicans* 109;	*Unpublished results*
19.	*C. albicans* 114;	*Table 6*
20.	*C. albicans* 117;	*Unpublished results*
21.	*C. albicans* 125.	*Table 6*

22. *C. albicans* 142; *Unpublished results*

23. *C. albicans* 185; *Table 6*

24. *C. albicans* 266; *Table 6*

25. *C. albicans* 286. *Table 6*

26. *C. albicans* 299; *Table 6*

27. *C. albicans* 366; *Unpublished results*

28. *C. albicans* 387. *Table 6*

29. *C. albicans* 388; *Unpublished results*

30. *C. albicans* 391. *Unpublished results*

31. *C. albicans* 395 *Table 6*

32. *C. albicans* 444; *Unpublished results*

33. *C. albicans* 472; *Unpublished results*

34. *C. albicans* 561; *Unpublished results*

35. *C. albicans* 1010; *Table 6*

36. *C. albicans* 1027; *Unpublished results*

37. *C. albicans* 1296; *Table 6*

38. *C. albicans* 2023; *Table 6*

39. *C. albicans* 2029 *Table 6*

40. *C. albicans* 2048; *Unpublished results*

41. *C. albicans* 2204; *Unpublished results*

42. *C. albicans* 2208; *Unpublished results*

43. *C. glabrata* 31; *Unpublished results*

44. *C. glabrata* 81; *Unpublished results*

45. *C. glabrata* 82; *Unpublished results*

46. *C. glabrata* 118; *Unpublished results*

47. *C. glabrata* 127; *Unpublished results*

48. *C. glabrata* 240; *Unpublished results*

49. *C. glabrata* 260; *Unpublished results*

50. *C. glabrata* 273; *Unpublished results*

51. *C. glabrata* 276; *Unpublished results*

52. *C. glabrata* 316; *Unpublished results*

53. *C. glabrata* 365; *Unpublished results*

54. *C. glabrata* 373; *Table 6*

55. *C. glabrata* 468; *Table 6*

56. *C. glabrata* 513; *Unpublished results*

57. *C. glabrata* 1104; *Unpublished results*

58. *C. glabrata* 1150; *Unpublished results*

59. *C. glabrata* 2181; *Unpublished results*

60. *C. glabrata* 2235; *Unpublished results*

61. *C. inconspicua* 1444; *Table 6*

62. *C. krusei* 9; *Unpublished results*

63. *C. krusei* 102; *Table 6*

64. *C. krusei* 268; *Unpublished results*

65. *C. palmioleophila* 4; *Table 6*

66. *C. palmioleophila* 368; *Table 6*

67. *C. palmioleophila* 370; *Table 6*

68. *C. palmioleophila* 377; *Table 6*

69. *C. palmioleophila* 405; *Table 6*

70.	*C. parapsilosis* 101;	*Unpublished results*
71.	*C. parapsilosis* 381;	*Unpublished results*
72.	*C. parapsilosis* 441;	*Unpublished results*