Emerging Saccharomycotina yeast pathogens

Detection and susceptibility profiles

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CHAPTER 1

General introduction

INTRODUCTION

The kingdom Fungi is a major domain of life and its representatives affect many aspects of human beings. Currently there are eight phyla, 12 subphyla and 46 classes recognised within the kingdom (Spatafora *et al.,* 2018). The phylum Ascomycota includes the subphylum of our interest and focus of this thesis, the Saccharomycotina, which mainly comprises of yeasts. The yeasts of the subphylum Saccharomycotina are part of everyday life of humans; they are found as commensals on the human body, they are used in industry for the production of bread, beverages, spirits, fabrics and much more. However, they exhibit a dark side as they are also known as human opportunistic pathogens (Kurtzman *et al.,* 2011). More specifically, the antifungal susceptibility profiles of clinically prevalent Saccharomycotina species have been analysed and studied in the light of their phylogeny; results of antifungal susceptibility testing of uncommon but emerging Saccharomycotina yeasts causing candidemia are presented; the synergistic effect of peptides and antifungal drugs on resistant Saccharomycotina yeasts is studied; the development of a novel multiplex qPCR approach for the detection of 25 common and uncommon *Candida* species is presented; proof of concept for a qPCR for the detection of members of the genus *Malassezia* is provided; the investigation of *Candida albicans* genotypes and their relation to Irritable Bowel Syndrome is addressed; and lastly we propose a protocol to correct misidentifications present in public databases.

The polyphyletic origin of yeasts

According to Kurtzman *et al.* (2011) the organisms considered as yeasts are fungi that asexually reproduce by budding or fission and that do not form their sexual states within or upon fruiting bodies. Now, the group of yeasts does not only include the ones belonging to the Saccharomycotina clade, but also Basidiomycota yeasts, e.g. species of *Malassezia*, *Cryptococcus* and *Trichosporon*, some of which are also important human and animal pathogens, however, they fall outside the scope of the current thesis.

The vast majority of yeasts related to human disease are Saccharomycotina yeasts, collectively called *Candida*. The “genus” *Candida* is an artificial genus, which derives from the times that taxonomy and identification was based on morphology, biochemical tests, fermentation and other physiological tests. Molecular phylogenetic methods showed and confirmed earlier beliefs that the “genus” *Candida* is highly polyphyletic and nowadays there is discussion about its reclassification and change of names for a vast amount of yeast species that are still called *Candida* (Borman & Johnson, 2021; Tsui *et al.,* 2008). An example is one of the major human pathogens, [*Candida*] *glabrata,* which is a member of the Nakaseomyces clade and phylogenetically distant from the Lodderomyces clade that is considered to be the “true” *Candida* clade as it includes the type species of the *Candida* genus, *Candida tropicalis* CBS 2310, type strain of *Candida vulgaris* Berkhout. Figure 1 shows the phylogenetic relationships of clinically important Saccharomycotina yeasts and closely related species. In this figure, the polyphyletic origin of such yeasts and the different lineages are clear. The most well-known Saccharomycotina opportunistic pathogens belong to the CTG clade, i.e. *C. albicans*, *Candida parapsilosis*, *C. tropicalis*, *Candida dubliniensis*, the Nakaseomyces clade, i.e. *C. glabrata,* and the Pichia clade with *Pichia kudriavzevii* (= *Candida krusei*). Those three clades are phylogenetically distant and the hypothesis is that pathogenicity in each of the clades has evolved independently for each of them (Gabaldón *et al.,* 2016), but there are more clades that include opportunistic pathogens that we will discuss within the scope of this thesis. However, there are no clades that consist only of opportunistic yeast pathogens.

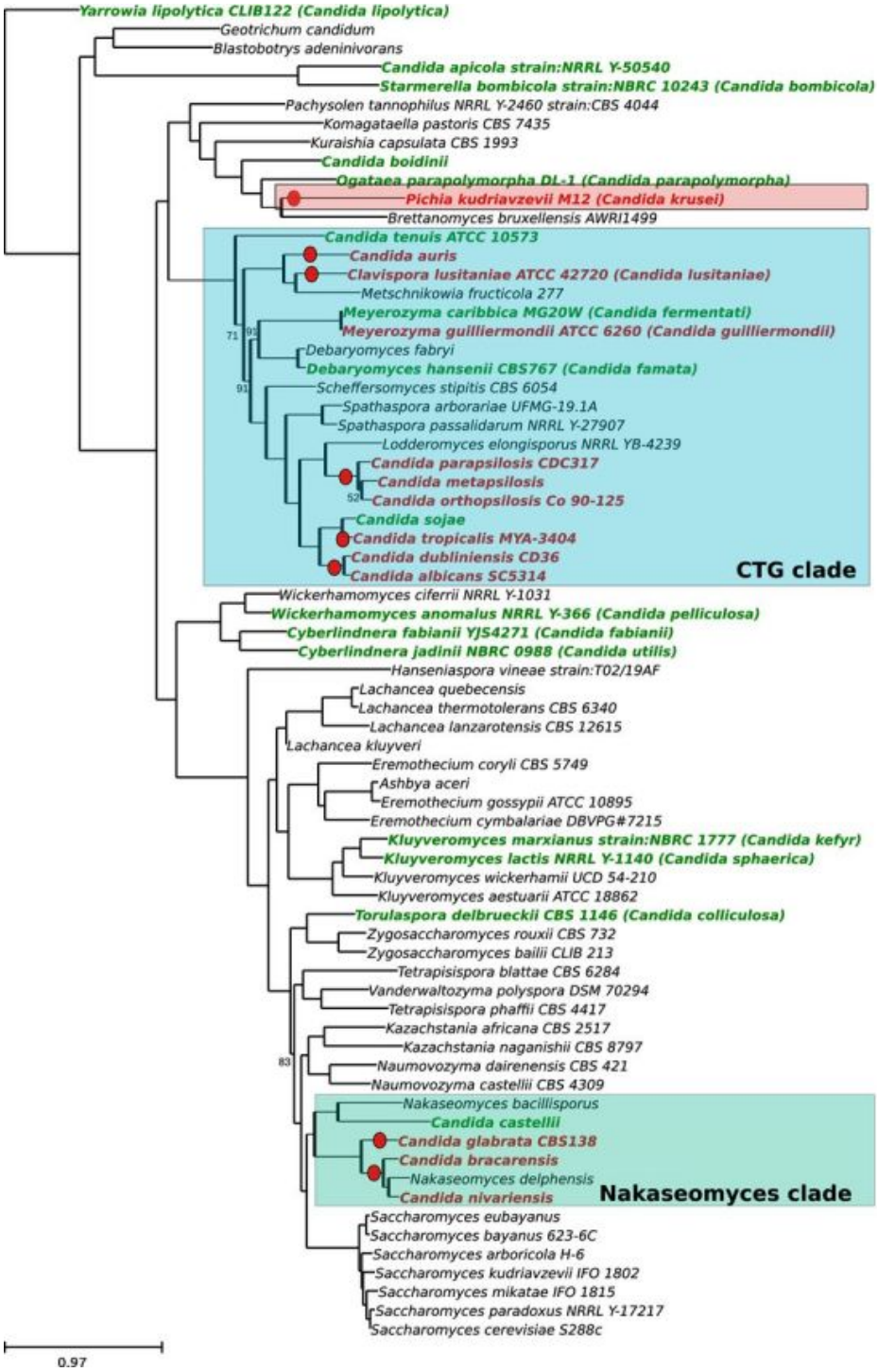

Figure 1. Phylogenetic relationships among *Candida* species based on 516 single copy genes. For *Candida* species, red-colored leaves indicate pathogenic species while green-colored leaves indicate non-pathogenic species (Gabaldón *et al*., 2016).

Reclassification and taxon name changing is not always received well, especially not by physicians involved in medical mycology (Kidd *et al.,* 2021). However, taxonomy and nomenclature are essential as many clades show intrinsic resistant patterns, e.g. fluconazole resistance in the Pichia clade, or the presence of molecular mechanisms that allow rapid emergence of resistance, e.g. *Candida auris*, as we show in this thesis (Schmalreck *et al.,* 2014; Stavrou *et al.,* 2019).

Yeasts occurring on humans - Commensals or pathogens?

Although the scope of this thesis is the detection of pathogenic yeasts and their antifungal susceptibility profiles, most yeasts are not true pathogens, at least the ones belonging to the Saccharomycotina. True pathogenic yeasts are for instance those formed by the dimorphic Ascomycota fungi in the order Onygenales, e.g. *Coccidioides immitis* and *Coccidioides posadasii*. Other Onygenales species, such as species of the genera *Blastomyces*, *Histoplasma*, and *Paracoccidioides*, are also causes of infections in human and they exhibit both a yeast and a filamentous form (Jiang *et al.,* 2018).

A weakened immune status is commonly referred to as immunosuppression or immunodeficiency, which are two different conditions. The first refers to a weakened condition of the immune system deriving from a certain drug regime, such as use of corticosteroids or a cancer treatment, and the second refers to different types of inherited or acquired diseases that weaken the immune system, such as primary T-cells immunodeficiencies or infection caused by Human Immunodeficiency Virus (HIV), respectively. Moreover, different yeasts are usually associated with different types of immunosuppression/immunodeficiency (Lanternier *et al.,* 2013; Georgiadou *et al.,* 2017). An example is *Cryptococcus neoformans* [sensu stricto], a basidiomycetous yeast, which is directly associated with causing central nervous system infections to individuals with a HIV infection/Acquired Immunodeficiency Syndrome (AIDS) and who do not receive antiretroviral treatment (Lin *et al.,* 2015). Representatives of the genus *Malassezia* are associated with causing sepsis to low-birth-weight neonates (Gaitanis *et al.,* 2012).

Opportunistic yeasts, however, are not always a cause of concern. The most well-known yeast apart from *Saccharomyces cerevisiae*, *Candida albicans*, is a human commensal which is an essential part of the human skin, certain mucosa (vagina and mouth) and the gut mycobiomes (Calderone and Clancy, 2011; Musumeci *et al.,* 2021). However, *C. albicans* is well known as the most common opportunistic yeast associated with human disease (Pappas *et al.,* 2018). The infections caused by *C. albicans* vary from uncomfortable skin conditions to life-threatening invasive infections. They can cause superficial infections, e.g., oropharyngeal candidiasis, vaginal candidiasis, penile candidiasis, cutaneous candidiasis, nail infections, and chronic mucocutaneous candidiasis. More importsant is that they can also cause invasive candidiasis, e.g., acute disseminated candidiasis and candidemia, chronic disseminated candidiasis, lower urinary tract candidiasis, renal candidiasis, respiratory tract candidiasis, osteomyelitis and arthritis, peritonitis, endocarditis, myocarditis and pericarditis, central nervous system candidiasis, endophthalmitis and choriotenitis, oesophagitis, gastrointestinal candidiasis, and intrauterine candidiasis (Richardson & Warnock, 2011). All these manifestations are not specifically caused by *C. albicans*. The causative agent may be any Saccharomycotina species called *Candida* and there may be other manifestations apart from the ones listed here, such as otomycosis or keratomycosis. Moreover, there is evidence that Saccharomycotina species, mainly *C. albicans*, might be involved in other aspects of human health, such as Irritable Bowel Syndrome (IBS). *C. albicans* is abundant in the human gastrointestinal system and there is research as

early as 1992 looking into the load of *C. albicans* between IBS patients and healthy individuals, alas without establishing a connection or finding *C. albicans* as the aetiology of IBS (Middleton *et al.,* 1992). Other reports, including our own research show that the presence of *C. albicans*, even though it might exhibit a high load and be among the most common yeast in fecal samples of IBS patients, is not directly connected to symptoms that those patients commonly experience. It has been shown that an overload of *C. albicans* in the gastrointestinal system can cause symptoms to healthy individuals that are similar to those of IBS patients, e.g. diarrhoea (Das *et al.,* 2021). Investigations of the mycobiome in connection to gastrointestinal conditions have only recently started and there are many unknown aspects and discoveries to be unravelled, nonetheless it is certain that *C. albicans* in the human gut seems to play a crucial role to human health and well-being.

Opportunistic Saccharomycotina yeasts

Though the commonly used name for infections caused by Saccharomycotina yeasts is candidiasis, not all of those yeasts are *Candida*. The Saccharomycotina yeasts involved in human infections belong to many different clades within this subphylum. Undoubtedly, the most common species associated with infections is *C. albicans* that shows the highest prevalence amongst the general human population (Arendrup, 2013). *C. albicans* is a representative of the Lodderomyces clade, which also includes other species commonly associated with candidiasis, though with a lower prevalence than *C. albicans*. Those species are *C. dubliniensis*, *C. tropicalis* and the *C. parapsilosis* species complex that includes *C. parapsilosis*, *Candida metapsilosis* and *Candida orthopsilosis*. In addition, other species of the Lodderomyces clade have been identified as uncommon causative agents of candidiasis, such as *Candida viswanathii* and *Lodderomyces elongisporus* (Shankarnarayan *et al.,* 2018; Stavrou *et al.,* 2019). The second most common causative agent of candidiasis in the general population is *C. glabrata*, that belongs to the Nakaseomyces clade and is a species distantly related to the Lodderomyces clade (Kurtzman *et al.,* 2011). Other Nakaseomyces representatives associated with candidiasis are species closely related to *C. glabrata*, namely *Candida nivariensis* and *Candida bracarensis* and they exhibit a low prevalence. *P. kudriavzevii* (=*C. krusei*), belonging to the Pichia clade is also commonly involved in human infections (Gong *et al.,* 2018). Another representative of the Pichia clade, which has been found lately to cause apparent nosocomial outbreaks is *Pichia norvegensis*. Both these species are intrinsically resistant to fluconazole and exhibit high minimum inhibitory concentrations (MIC) to other tri-azoles, similar to other emerging members of the Pichia clade, such as *Candida inconspicua* (Borman *et al.,* 2019; Díaz-García *et al.,* 2019). Another, commonly detected agent of candidiasis is *Meyerozyma guilliermondii* (= *Candida guilliermondii*). The Meyerozyma clade also includes *Meyerozyma caribbica* (= *Candida fermentati*), a less common agent of candidiasis that similar to *M. guilliermondi* shows decreased susceptibility to azoles and echinocandins (Al-Sweih *et al.,* 2015; Morita *et al.,* 2018).

Clavispora lusitaniae (= *Candida lusitaniae*) is another commonly found yeast species associated with candidiasis and a member of the Metschnikowiaceae family. This family includes a number of species apart from *Cl. lusitaniae* that have been identified as a cause of infection in humans, such as the closely related *Candida intermedia*. Interestingly enough, the infamous *Candida auris* is a member of the same family, however it forms its own clade within the Metschnikowia family where it sits next to the *Candida haemulonii* species complex and it has been shown that this clade is prone to acquire resistance to several antifungal drug classes. On the contrary, *Cl. lusitaniae* and *C. intermedia* do not commonly exhibit increased susceptibility to antifungals.

The clades mentioned, usually include the most common species associated with candidemia. However, there is a wide variety of other species deriving from multiple clades of the Saccharomycotina associated with candidiasis, namely *Kluyveromyces marxianus* (= *Candida kefyr*) in the Kluyveromyces clade, *Wickerhamomyces anomalus* (= *Candida pelliculosa*), *Cyberlindnera jadinii* (= *Candida utilis*) both from the Wickerhamomycetaceae, *Debaryomyces hansenii* (= *Candida famata*) in the Debaryomyces clade, *Wickerhamiella pararugosa* (= *Candida pararugosa*) in the Wickerhamiella clade, *Diutina rugosa* (= *Candida rugosa*) in an ambiguous clade recently assigned within the Metschnikowia family, and *Yarrowia lipolytica* (= *Candida lipolytica*) in the basal Yarrowia clade of the Saccharomycotina, are the ones identified as such in this thesis with a significant prevalence (Stavrou *et al.,* 2019). The prevalence of the different species might be circumstantial, geographical, related to the patient group, or due to still unidentified reasons. Table 1 (adapted from Stavrou *et al.,* 2019, Chapter 2) shows the species usually involved in candidemia.

Identification and diagnosis of opportunistic yeast infections

Identification of opportunistic yeasts has been a subject of debate for a long time. Opportunistic yeasts are hard to detect and identify, especially from clinical samples, but there has been a long discussion which nomenclature to use as this often results in confusion on the identification (Borman & Johnson 2021; Kidd *et al.,* 2021; Wiederhold and Gibas, 2018). Firstly, detection of yeast pathogens is a challenging matter. There are well-known challenges, such as use of appropriate culturing media, long incubation times needed and low amounts of the infectious agent present in clinical samples obtained from humans and animals. For fungal and yeast cells, the challenges are even greater due to the unique cell structure of the cell with a chitin-rich cell wall that is hard to break to release the DNA, the confusing nomenclature, and the fact that their presence in a sample does not always hint to an infection as certain species occur also as commensal (Wickes & Romanelli, 2020).

For years, the gold standard for identification of pure cultures has been PCR that targets specific DNA markers with the ribosomal DNA Internal Transcribed Spacer (ITS) as the most widely used marker. The ITS region is an excellent, multicopy DNA marker that in most of the cases gives a definite identification. Many ITS sequences are present in DNA databases such as NCBI GenBank, which is the most commonly used database, and these can be used as a reference in order to identify an unknown isolate (Pincus *et al.,* 2007). Also, the ISHAM-ITS reference DNA barcoding database contains ITS sequences for human and animal pathogenic fungi and importantly enough is a curated database (Irinyi *et al.,* 2015). Unfortunately, some public databases are not curated implying that there are numerous wrong entries that can lead to misidentifications which subsequently can impact the diagnosis, the choice of drug and essentially the outcome for the patient (Stavrou *et al.,* 2018).

Filamentous fungi of clinical importance may be easier to identify in a culture, as they often possess characteristics easily distinguishable by microscopy, which is not easily applied to Saccharomycotina yeasts. Although, yeasts also exhibit certain characteristics distinguishable by microscopy, for example, the germ tubes that *C. albicans* produces under starvation conditions, but those are not reliable enough to result in a definite identification. Chromogenic media have been used successfully for the identification of the most common pathogenic yeasts, e.g. *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *P. kudriavzevii*. These methods suffer from limitations, namely poor sensitivity and, importantly, uncommon but emerging yeasts species in the clinic cannot be distinguished

Table 1. Prevalence of candidemia related Saccharomycotina species (in alphabetical order) from clinical samples as identified from worldwide epidemiology literature. In the table, the lowest and highest prevalence of each of the species found along with the average prevalence of the mentioned species from every published article used in the meta-analysis (modified table from Stavrou *et al*., 2019).

Species overview	Average Prevalence	Max. Prevalence	Min. Prevalence
Candida albicans	46.6%	70%	20.9%
Candida bracarensis	0.4%	0.8%	<0.01%
Candida dubliniensis	1.8%	4.4%	0.6%
Candida glabrata	16.7%	30%	1.9%
Candida inconspicua	0.1%	0.3%	<0.01%
Candida intermedia	0.3%	0.6%	<0.01%
Candida nivariensis	0.07%	0.2%	<0.01%
Candida orthopsilosis	1.4%	4%	<0.01%
Candida palmioleophila	0.1%	0.3%	<0.01%
Candida parapsilosis	16%	45.4%	3.3%
Candida tropicalis	12%	41.6%	5.0%
Clavispora lusitaniae (= *Candida lusitaniae*)	1.3%	2.8%	0.6%
Cyberlindnera jadinii (= *Candida utilis*)	0.1%	0.3%	<0.01%
Debaryomyces hansenii (= *Candida famata*)	0.1%	0.4%	<0.01%
Diutina rugosa (= *Candida rugosa*)	0.7%	3.2%	<0.01%
Kluyveromyces marxianus (= *Candida kefyr*)	0.4%	1.3%	<0.01%
Meyerozyma caribbica (= *Candida fermentati*)	0.2%	0.6%	<0.01%
Meyerozyma guilliermondii (= *Candida guilliermondii*)	1.9%	6.5%	0.2%
Pichia kudriavzevii (= *Candida krusei*)	2.4%	6.2%	1.0%
Pichia norvegensis (= *Candida norvegensis*)	0.04%	0.1%	<0.01%
Wickerhamiella pararugosa (= *Candida pararugosa*)	0.5%	1%	<0.01%
Wickerhamomyces anomalus (= *Candida pelliculosa*)	0.1%	0.5%	<0.01%
Yarrowia lipolytica (= *Candida lipolytica*)	0.4%	0.8%	<0.01%

by chromogenic media. There are steps taken towards the development of chromogenic media that can identify other yeasts apart from those most commonly found in the clinic, such as *C. auris* that has become a threat to public health during the last decade (de Jong *et al.,* 2021). Regarding the use of media in relation to opportunistic Saccharomycotina yeasts, there is usually no special media requirement for those. Sabouraud Dextrose medium supplemented with chloramphenicol that is used widely in most laboratories, allows growth of most yeast species, but not *Malassezia* yeasts. Regarding the growth conditions we noticed that some yeasts that infect humans who have an average body temperature of 37°C, do not all grow or slower or not at all at this temperature. For these reasons, culturing may require a prolonged time in order to achieve growth *in vitro* (e.g., 24-72h to obtain a culture), and this is suboptimal for the detection and identification of opportunistic yeast pathogens, as candidemia cause critical illness and a fast turn-around time is crucial to cure a patient.

The most commonly used techniques for species identification are currently PCR-based barcode sequencing and mass spectrometry-based, viz. Matrix-Assisted Laser Desorption - Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS), whereas

antibody-related tests are less commonly used and they focus more on detection than identification. Figure 2 summarises the advantages and disadvantages of the methods commonly and less commonly used for the detection and identification of yeast pathogens (Consortium OPATHY and Gabaldón, 2019). As shown, the vast majority are nucleic acid-based identification methods from which many, apart from next-generation sequencing (NGS) and PCR coupled with magnetic resonance, are commercially available. The nucleic acid-based methods are, apart from next-generation sequencing, not expensive. However, only PCR, qPCR and NGS are species-targeted methods which is an important aspect for an identification assay considering the phylogenetic variety of Saccharomycotina species that may be responsible for an infection. Antibody-based detection methods have all the same characteristics; several are commercially available, they offer species-targeted identification and they can be used directly on patient material. The downside is that those methods are not as sensitive as nucleic-based methods, such as qPCR or NGS. From

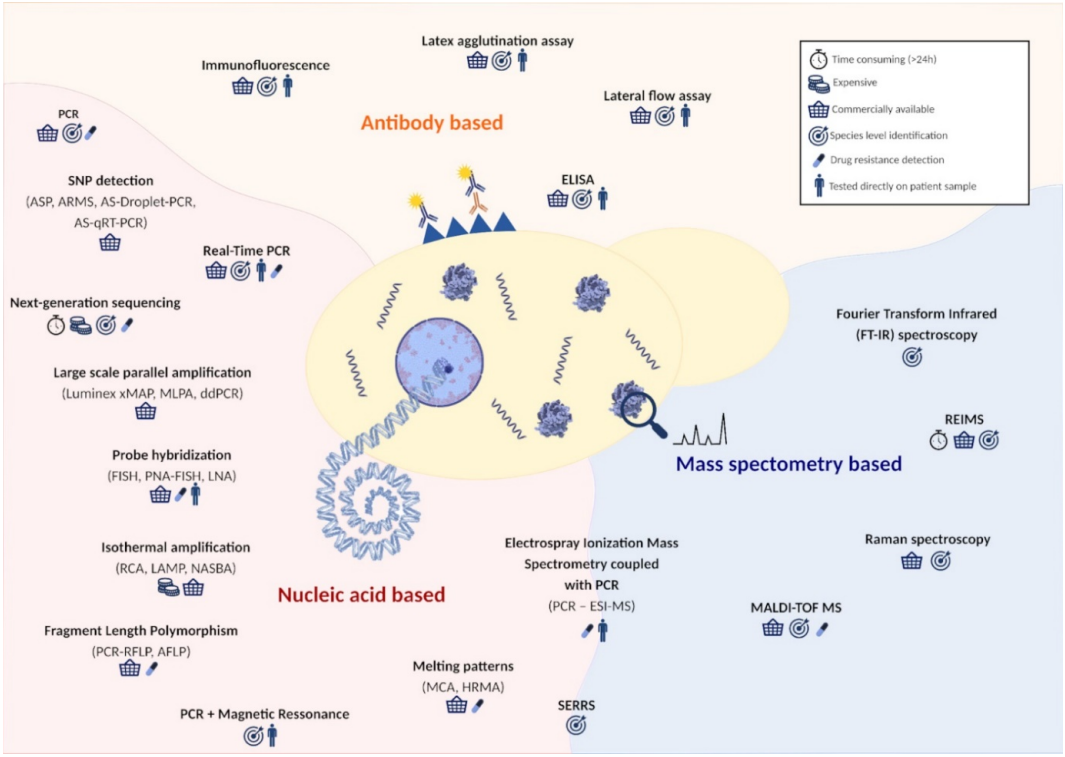

Figure 2. Overview of approaches for the detection of fungal pathogens. Schematic representation of the different technologies used for the identification of fungal organisms. These techniques can be based on mass spectrometry (blue background), nucleic acid (red background) or antibody (orange background). Techniques based on more than one of these aspects are represented in the border of the respective divisions. ASP—allele-specific PCR; ARMS—amplification refractory mutation system; AS-Droplet-PCR—combination of ASP with droplet PCR; AS-qRT-PCR—combination of ASP with quantitative PCR; MLPA—multiplex ligation-dependent probe amplification; ddPCR—droplet digital PCR; FISH—fluorescent in situ hybridization; PNA-FISH—peptide nucleic acids-FISH; LNA—locked nucleic acids; RCA—rolling-circle amplification; LAMP—loop-mediated isothermal amplification; NASBA—nucleic acid sequence-based amplification; RFLP—restriction fragment length polymorphism; AFLP—amplified fragment length polymorphism; ELISA—enzyme-linked immunosorbent assay; MALDI-TOF MS—matrix-assisted laser desorption-time of flight mass spectrometry; PCR-ESI-MS—electrospray ionization mass spectrometry coupled with broad-spectrum PCR; SERRS—surface-enhanced resonance Raman spectroscopy; MCA—melting curve analysis; HRMA—high-resolution melting analysis; REIMS—rapid evaporative ionization mass spectrometry (from Consortium OPATHY and Gabaldón, 2019).

the mass spectrometry-based methods, MALDI-TOF MS is the most commonly used as it is commercially available, Food and Drug Administration (FDA)-approved and is CE/IVD, it provides species-level identification and, importantly, it may provide resistance-related information (Consortium OPATHY and Gabaldón, 2019). The downside is that it cannot be used directly on patient material. Real-time PCR (qPCR)-based methods became more widely used as they have several advantages, such as high sensitivity and specificity; they can be directly used with patient material thus skipping any prior, lengthy culturing step; they have considerable lower costs than other methods with similar sensitivity and specificity, such as NGS.

In addition, qPCR has the possibility for multiplexing making it possible to detect and differentiate as many as six different targets within a single reaction (Consortium OPATHY and Gabaldón, 2019; Chapter 5). Though multiplexing is laborious and challenging, it is a good way to test the limited amount of patient material in cases that a definite diagnosis is needed; especially in cases of candidemia where the causative agent could be one of many that do not all respond to the same treatment.

Antifungal susceptibility profiles of Saccharomycotina yeasts and antifungal drugs

The implication of candidemia caused by less commonly occurring species is mainly the lack of knowledge of their antifungal susceptibility profiles. Scientific literature provides *in vitro* susceptibility data for several uncommon species, but the majority of information comes from case reports (Desnos-Ollivier *et al.,* 2012, 2021; Pérez-Hansen *et al.,* 2019; Stavrou *et al.,* 2020).

There is ample amount of data on the antifungal susceptibility patterns for the five clinically most common yeast species of Saccharomycotina and there is a trend to include more uncommon yeasts (Espinel-Ingroff *et al.,* 2021). Clinical break points (CBPs) and/or epidemiological cut off values (ECOFFs) have been established for *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *P. kudriavzevii* according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Table v. 9.0: http://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/). In addition, Clinical & Laboratory Standards Institute (CLSI) sets ECOFFs for the same species, and *M. guilliermondii* and *Cl. lusitaniae*. For these two latter species, however, the information is incomplete compared to the first five species and therefore the ECOFF and CBP values for *M. guilliermondii* and *Cl. lusitaniae* are not strictly set (Stavrou *et al.,* 2020). For rare species, ECOFFs for both international standard broth dilution methods (EUCAST and CLSI) are missing due to the lack of a statistically significant number of cases that is needed to set reliable ECOFFs.

For clinically uncommon species, the antifungal regimen used to treat infections was based on empirical treatment rather than official guidelines and only recently official guidelines for the treatment of rare yeast infections have been published (Chen *et al*., 2021). Due to the rarity of these species, it is difficult to collect a sufficient amount of information to guide a treatment. In general, there is evidence that rare Saccharomycotina yeasts show antifungal susceptibility patterns, which are related to their phylogenetic position (Lin *et al.,* 2015; Pérez-Hansen *et al.,* 2019; Stavrou *et al.,* 2019). Moreover, this trait seems to be clade specific (Stavrou *et al.,* 2019). This implies that when a rare species is identified as the cause of an infection, potentially the same antifungal drugs will be effective against it as the ones used for relatives that are more common and for which antifungal susceptibility data already exist (Stavrou *et al.,* 2019). For instance, *C. glabrata* shows high MIC values for fluconazole, however for its clinically less common relatives, namely

C. nivariensis and *C. bracarensis*, there are no official guidelines on the susceptibilities to antifungal drugs. Nevertheless, due to their phylogenetic position and clinical evidence, it is known that they also exhibit high MIC values to fluconazole (Desnos-Ollivier *et al.,* 2021; Desnos-Ollivier *et al.,* 2012; Pappas *et al.,* 2016). As a rule of thumb, this phylogeny-based information can be used for other rare species when involved in infections and when the data available are not enough to decide on a treatment option. However, it should be noted that this applies only when we are referring to intrinsic antifungal susceptibility patterns. For instance, acquired resistance has been detected in a large number of *C. glabrata* isolates that became resistant to echinocandins, mainly in the U.S.A. because of prior treatment with echinocandins (Pappas *et al.,* 2016).

Nonetheless, inference of phylogeny-based antifungal susceptibility patterns could be useful for the majority of cases and, due to lack of official guidelines, choosing a treatment in line with the common susceptibility pattern of a phylogenetically related species can be a temporal solution until sufficient data is collected for the less common yeast species. This is important as information on the susceptibility to antifungal drugs and the possible resistance mechanisms are of outmost importance for the treating clinician who must answer the question: “What is the optimal treatment option for this infection?”. The answer to this question is a combination of knowing the identity of the species and their generic antifungal susceptibility profiles. The only straightforward solution to this is Whole Genome Sequencing (WGS), however we are still far from implementing this method in routine diagnostics (Consortium OPATHY and Gabaldón, 2019; Wickes & Romanelli, 2020).

If possible, infections caused by *Candida* species are treated with antifungal drugs that are recommended against the species following the guidelines of EUCAST and/or CLSI. The choice of the appropriate drug is also related to the kind of infection caused, i.e. superficial or systemic, the susceptibility of the species responsible for the infection, and the condition of the individual patient (Pappas *et al.,* 2016). Specific antifungal drugs are more effective against certain *Candida* species because of the intrinsic or acquired resistance that those species or strains possess. Intrinsic or primary resistance refers to the wild type resistance of a species, whereas acquired or secondary resistance refers to non-wild type strains or isolates which evolved to become resistant against drugs to which the wild type is susceptible (Ksiezopolska & Gabaldón, 2018). Our research focused on *in vitro* testing of systemically used antifungal drugs, e.g. the tri-azoles, fluconazole, voriconazole, itraconazole, posaconazole, the echinocandins, anidulafungin, micafungin and caspofungin, and last but not least the polyene amphotericin B for a number of rare, but clinically emerging yeasts. In addition to the above, there are other drug classes and drugs used for the treatment of yeast and fungal infections. However, these are applied topically and used to treat superficial or mucocutaneous conditions and they are not suitable for the treatment of systemic yeast infections. Examples are allylamines that are mainly used for the treatment of dermatophytosis; imidazole compounds that are mainly used as a topical solution, e.g. ketoconazole, clotrimazole, miconazole nitrate; nystatin and natamycin, which belong to the class of polyenes and are used for topical treatment; and flucytosine, a synthetic fluorinated analogue of cytosine, which is used to treat systemic infections but has a limited mode of action against *Candida* species (Richardson & Warnock, 2011).

Antimicrobial peptides as an alternative antifungal treatment

It is clear that drug resistance of clinically important yeasts will become a major issue in the future. The example of *C. auris* is the most prominent and relevant illustration that emerged during the last few years (Forsberg *et al.,* 2019). This yeast associated with causing infections

in humans and that has recently been cultured from sea water nowadays exhibits resistance to multiple classes of antifungal drugs and a limited number of isolates were found to be even multidrug resistant, rendering treatment extremely challenging, if not impossible (Ademe & Girma, 2020; Arora *et al.,* 2021; de Jong & Hagen, 2019). The need for novel classes of antifungal drugs has been known to clinicians and mycologists for years and pharmaceutical companies recently show a renewed interest towards this goal (Roemer & Krysan, 2014). There is an immediate need for substances that do not only exhibit antifungal action, but also do not promote resistance-related mutations leading to the development of resistance (Perfect, 2017). Antimicrobial peptides (AMPs) fall within this category. These AMPs are naturally derived peptides used by organisms as a first-line defence against pathogens. Since they were first identified, extensive research has been conducted on how to implement AMPs in the fight against pathogens (Bondaryk *et al.,* 2017). Related to yeasts, AMPs can be successfully used against opportunistic yeasts, such as *C. auris* (Pathirana *et al.,* 2018). Moreover, when used in combination with antifungal drugs they can render a previously resistance isolate more sensitive, making it possible again to treat previously untreatable yeast infections (Chapter 4). In addition, AMPs, such as human lactoferrin 1-11 [hLF(1-11)] that is derived from a naturally produced human AMPs, pose no significant risk for the patient (Brouwer *et al.,* 2018). With all those unique characteristics, AMPs could be a possible answer to the problem of emerging resistance and uncommon yeast species; however, there is still a long way to go in this research area (Buda De Cesare *et al.,* 2020).

Outline of this thesis

In this thesis, we studied the correlation between phylogenetic relationships of Saccharomycotina yeasts and their intrinsic antifungal susceptibility patterns. With this task we also encountered a list of emerging Saccharomycotina yeasts worldwide for which antifungal susceptibility data are either scarce or incomplete and we identified the need for an identification tool which will simultaneously provide an identification and provide information about treatment options. Finally, yet importantly, the need for novel antifungal agents has been apparent and we investigated how AMPs can be used against common and emerging Saccharomycotina opportunistic pathogens.

The thesis consists of seven chapters. Chapter 2 is a meta-analysis that focus on identifying the global prevalence of *Candida* species causing candidemia and their antifungal susceptibility patterns. In this chapter, we identified 23 prevalent species of *Candida* worldwide, including the five most common candidemia ‘‘culprits’’. Moreover, the list was extended to other less common species, which are found to cause candidemia worldwide, such as *C. auris* that in the last decade has been a continuous cause of nosocomial outbreaks. The antifungal susceptibility patterns to azoles and amphotericin B correlated to the phylogeny of the species and this served as a backbone to present how closely related species exhibit similar intrinsic antifungal susceptibility patterns.

Chapter 3 is a continuation of Chapter 2. We realised that antifungal susceptibility data for uncommon Saccharomycotina are scarce. Therefore, we tested up to 30 isolates for 23 species identified in Chapter 2, for their susceptibility to systemically used antifungal agents, namely amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, anidulafungin, micafungin and caspofungin according to the EUCAST broth microdilution method. The results were as expected based on our analysis in Chapter 2. The correlation between phylogeny and susceptibility patterns was reinforced and, importantly, we generated antifungal susceptibility data for multiple isolates of uncommon species that were previously not available or were available only for single or few isolates of a species.

Chapter 4 described the use of AMPs against Saccharomycotina yeasts that cause candidemia. We tested *in vitro* a lactoferrin derived AMP, hLF(1-11), against the Saccharomycotina species exhibiting high minimum inhibitory concentrations (MICs) to fluconazole and/or anidulafungin. Initially, we tested the susceptibility to fluconazole and anidulafungin of 121 strains of 25 species with the broth microdilution method according to EUCAST. The strains that showed high MICs were further tested with hLF(1-11) and a combination of hLF(1-11) and the antifungal drug, either fluconazole or anidulafungin. The findings showed a synergistic combinatory effect of hLF(1-11) and the antifungal drug revealing that a resistant strain becomes more susceptible when hLF(1-11) is added. The different species used in this study form an important contribution towards illuminating the potential use of AMPs as an alternative or additive antifungal compound.

Chapter 5 presents and describes for the first time a phylogeny-driven multiplex qPCR assay for the detection of 25 *Candida* species associated with candidemia. This assay which is a hydrolysis probe-based qPCR assay includes three panels. The panels contain different *Candida* species and every panel can differentiate between 4 to 5 species or groups of species. The novelty of this assay is that every panel corresponds to species with a different antifungal susceptibility pattern. Panel 1 includes species that are intrinsically susceptible to all antifungals; Panel 2 contains species with intrinsically elevated MICs to azoles or with the ability to develop this; and Panel 3 covers species with high MICs to both azoles and echinocandins, such as the infamous *C. auris.* This assay has the potential to be used directly with patient material as its limit of detection was notably low, namely 1-7 cells/reaction.

Chapter 6 presents a proof-of-concept qPCR assay for the detection of the basidiomycetous yeast genus *Malassezia*. To our knowledge this is the first attempt to study a hydrolysis probe-based qPCR approach for the detection of members of this genus, which contains opportunistic pathogens associated with sepsis in low birth neonates, and it is largely underestimated as its species have specific nutritional requirements, namely lipid dependency. This assay showed a low limit of detection 4-40 cells/reaction depending on the *Malassezia* species.

Chapter 7 investigated how different *C. albicans* genotypes are relevant to Irritable Bowel Syndrome (IBS) patients. The gut may also serve as a reservoir for candidemia and, hence, we studied a number of virulence-related phenotypes. It is long known that the microbiome and mycobiome may play a role in IBS, but different genotypes of *C. albicans* and their diversity between different individuals and how they impact individuals has never been studied so far. The findings do not reveal a direct link with IBS due to the limited number of samples studied. Nonetheless, this is a valuable contribution and the findings are worth to be further investigated.

Chapter 8 describes a case of misidentified data deposition in NCBI GenBank which evolved into a proposal on how to eliminate wrong entries in such public databases. A *C. albicans* isolate has been wrongly annotated as *Naumovozyma dairenensis*, a species which is only distantly related to *C. albicans* and that is not known to cause infections or reside in the human body. Furthermore, the niche where this yeast was found, namely the human gut, was unlikely to justify the presence of *N. dairenensis*. Furthermore, other misidentification cases in NCBI GenBank were found and these can strongly impact the work of scientists as their research largely depends on the use of public databases. Hence, we proposed a protocol that public databases can adopt to eliminate such errors.

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