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João Luís Cruz Prates

Effect of fetal bovine serum on *Candida glabrata* cultures

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biomédica
Ramo de Engenharia Clínica

Trabalho efetuado sob orientação da
Professora Doutora Mariana Contente Rangel Henriques
Universidade do Minho

e do
Professor Doutor Patrick Van Dijck
KU Leuven

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DECLARAÇÃO

Nome: João Luís Cruz Prates

Endereço eletrónico: joaoprates.biom@gmail.com

Telefone: 910357794/259939562

Número do Cartão de Cidadão: 13461633

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Orientador/a/es:

Professora Doutora Mariana Contente Rangel Henriques

Professor Doutor Patrick Van Dijck

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To my maternal grandfather and my paternal grandmother,
This one I really need to share with both of them, they would be proud of me.

ABSTRACT

Candida glabrata, is a yeast that takes part of the human's microbial flora as an opportunistic pathogen and has been emerging, becoming an important and dangerous nosocomial pathogen.

The main evidence that raised the development of this project was the observation that there was a secretion of unknown composition identified on *C. glabrata* cultures grown in the presence of fetal bovine serum (FBS). However, the results showed that all the strains tested from *C. glabrata*, *Saccharomyces cerevisiae* and *Candida albicans* produced secretion only in the presence of FBS, what was not expected. The goals were to evaluate the role of FBS in *C. glabrata* secretion production, to characterize its composition and to evaluate its effect in *C. glabrata*'s virulence factors.

It was confirmed by SDS-PAGE that the secretion only occurred in the presence of FBS. In addition, it was shown that the proteins on the secretion were not part of the proteic composition of the FBS but came from the cells. The way the cells were affected by the presence of the FBS is still unclear. By mass spectrometry, five genes were found on the secretion that had homology on *S. cerevisiae* cell wall glucanases.

As the origin of the secretion and the composition of that secretion were already investigated, next a deletion collection was screened to try to determine the gene(s) responsible for the secretion. However, there were no correlation between the mutants and the production of secretion.

A susceptibility test was performed for fluconazole and it was possible to verify a morphological difference between the cells grown in FBS and without it and a MIC value higher in the presence of FBS. The way the FBS affects the MIC is not elucidated. Congo red and calcofluor white were also tested in the presence of FBS, but, the results were inconclusive as the FBS did not have any remarkable effect on the two cell wall stress dyes tested.

Regarding adhesion, it was possible to verify that with increasing concentration of FBS, the cell adhesion decreased.

In conclusion, the secretion in the presence of FBS is not exclusive for *C. glabrata* cultures however, this study can bring new insights on *C. glabrata* virulence.

RESUMO

A *Candida glabrata* é um fungo que faz parte da flora microbiana do organismo humano, sendo um agente patogénico oportunista, que se tem vindo a tornar um importante e perigoso patogeno nosocomial.

A evidência para este projeto surgiu da observação de que existia uma secreção de composição desconhecida nas culturas de *C. glabrata* com soro fetal bovino (FBS). No entanto, os resultados mostram que todas as estirpes de *C. glabrata*, *Saccharomyces cerevisiae* e *Candida albicans* que foram usadas produziram secreção na presença de FBS, o que não era esperado.

Os objetivos foram avaliar o papel do FBS na produção dessa secreção em culturas de *C. glabrata*, caracterizar a sua composição e avaliar o seu efeito nos fatores de virulência da *C. glabrata*.

Foi confirmado através de SDS-PAGE que a secreção apenas ocorria na presença de FBS. Para além disso, foi mostrado que as proteínas da secreção não faziam parte da composição do FBS mas tinham origem nas células. A forma como as células são afetadas pela presença do FBS ainda não é clara. Através de espectrometria de massa foram encontrados cinco genes na secreção com homologia nas glucanases da parede celular da *S. cerevisiae*.

Assim que a origem da secreção e a sua composição foram investigadas, uma "deletion collection" de *C. glabrata* foi cultivada na presença de FBS com o objetivo de determiner o(s) gene(s) responsáveis pela secreção. No entanto, não foi possível estabelecer uma correlação entre os mutantes e a produção da secreção.

Um teste de susceptibilidade ao fluconazole foi realizado e foi possível verificar a existência de diferenças morfológicas entre as células suplementadas por FBS e sem FBS e, para além disso, o valor do MIC aumentou na presença do FBS. A forma como o FBS afeta o MIC não está ainda determinada. O Congo red e o calcofluor white foram também testados na presença de FBS mas os resultados foram inconclusivos uma vez que o FBS não teve efeito em nenhum dos corantes testados.

Em relação à adesão, foi possível verificar que com o aumento da concentração de FBS, a adesão celular diminuiu.

Para concluir, a secreção na presença de FBS não é exclusiva para culturas de *C. glabrata*, no entanto este estudo pode trazer novos desenvolvimentos ao conhecimento da virulência da *C. glabrata*.

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Figure 8 - Image of the 96-well plate containing the MIC assay (24h) to test fluconazole. The first row of the plate is composed of empty wells. In the second to fourth rows there are cultures of *C. glabrata* wild type cells in SCD. In the fifth to seventh rows there are *C. glabrata* wild type cells in SCD plus FBS. The concentration of antifungal in both this groups of rows decreases from well to well till 0 (as according to the information on the first row). The last row of the plate contains the controls (no antifungal), constituted only by minimal medium with glucose (SCD Medium), *C. glabrata* cultured in minimal medium with glucose (SCD cells), minimal medium with glucose supplemented with FBS (FBS+SCD Medium) and *C. glabrata* cultured in minimal medium with glucose supplemented with FBS (FBS+SCD cells), respectively.

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ABBREVIATIONS

ABC: ammonium bicarbonate

ACN: acetonitrile

CR: Congo red

CSM: complete supplement mixture

CW: calcofluor white

DTT: dithiothreitol

ECM: extracellular matrix

FBS: Fetal bovine serum

GPI: glycosylphosphatidylinositol

HTL: *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT

IAA: iodoacetamide

MIC: minimal inhibitory concentration

MS: mass spectrometry

PBS: phosphate-buffered saline

Rpm: rounds per minute

SC: synthetic complete medium

SCD: minimal medium with glucose

SDS: sodium dodecyl sulfate

TE: tris-EDTA buffer

TFA: trifluoroacetic acid

UA: urea buffer

XTT: 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

YPD: yeast extract peptone dextrose

1. CHAPTER I

General Introduction

1.1 Motivation and Goals

Candida species are yeasts that are part of the human's microbial flora as opportunistic pathogens. After *Candida albicans*, *Candida glabrata* is the most prevalent yeast pathogen, with a high mortality rate in hospitals all around the world. One of the biggest problems associated with this increasing prevalence is the delay till the initiation of the right therapy (because *C. glabrata* is tolerant to the azole antifungals that are used to treat *Candida* infections).

The main evidence for this project was the observation that there was a secretion of unknown composition identified on *Candida glabrata* cultures in minimal medium with glucose and fetal bovine serum (FBS) (Figure 1) on solid medium and liquid medium.

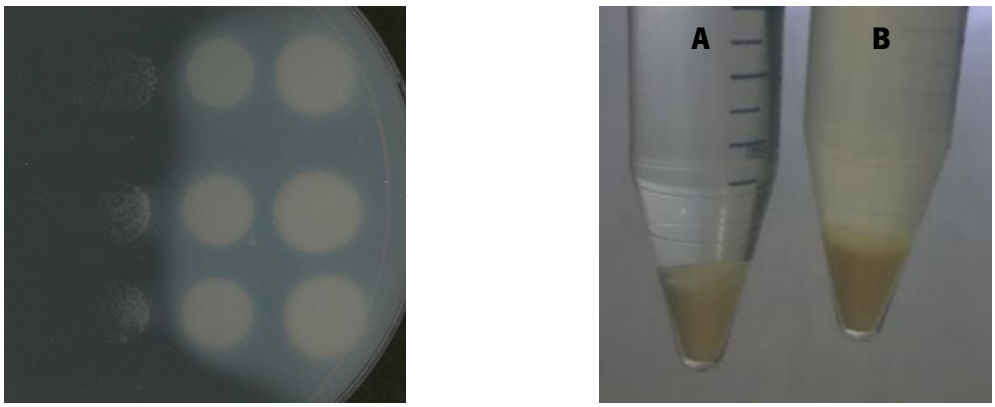


Figure 1 - The left picture illustrates several colonies of *C. glabrata* cultured in minimal medium with agar and supplemented with glucose and FBS; the right picture represents two falcons with cultures of *C. glabrata* cultured in minimal medium with glucose (A) and *C. glabrata* in minimal medium supplemented with glucose and FBS (B).

From the observation of the Figure 1 it is possible to identify a clear region around the cultures of *C. glabrata* spotted in solid minimal medium with FBS. That clear region can be observed also on the liquid cultures and with more evidence. Comparing with the culture of *C. glabrata* without FBS, the falcon with the culture of *C. glabrata* supplemented with FBS presents a layer of white secretion up to the pellet without the cells and a less clear supernatant layer.

So, the main purpose of this project was to determine if the secretion only occurred for *C. glabrata* and to evaluate the role of FBS in the secretion production. Moreover, it was also a goal to characterize the composition of that secretion. This analysis might be important for a better understanding of *C. glabrata* colonization and virulence mechanisms, and probably will give new important insights on this species that could be useful for the development in the future of more effective drugs against this pathogen.

1.2 State of Art

1.2.1 Nosocomial bloodstream infections

Nowadays, *Candida* is the fourth most common cause of nosocomial bloodstream infections (BSI) in the USA. *Candida albicans* is the most common *Candida* species causing BSI accounting for 54% of the cases. Secondly ranked is *Candida glabrata*, a species that is responsible for 19% of BSI infections in this country (Wisplinghoff et al., 2004). *Candida* is obviously an important cause of candidemia being nowadays an important and dangerous nosocomial pathogen (Pfaller et al., 2003; Trick et al., 2002). In the last three decades there was a significant increase in the cases of fungal infections in humans (Lass-Flörl, 2009), usually due to the use of invasive surgical procedures, immunosuppressive therapies and broad-spectrum antibiotics (Williams et al., 2011). These last two factors seem to increase in proportion with the number of infections caused by *C. glabrata* (Hajjeh et al., 2004). Over the last 15 years, the number of *C. glabrata* infections associated to BSI increased in North America (from only 12% of the *Candida* species, between 1992 and 1993, to 26% of the *Candida* species, between 2004 and 2008), while the number of *C. albicans* and other *Candida* (*Candida parapsilosis*, *Candida tropicalis* and *Candida krusei*) BSI cases stabilize (Table 1), allowing a better understanding of *C. glabrata* importance.

Table 1 - Temporal variation of bloodstream infections for different *Candida* spp. in North America (Pfaller & Diekema, 2010)

				% Total by *Candida* spp.*		
Location	Study period	Reference	No. of isolates	*Candida albicans*	*Candida glabrata*	Other *Candida*
United States	1992-1993	Kao et al. (1999)	837	52	12	35
United States	1993-1995	Pfaller et al. (2002a)	79	56	15	25
United States	1995-1997	Pappas et al. (2003)	1,593	46	20	28
United States	1995-1998	Pfaller et al. (2002a)	934	53	20	25
United States	1998-2000	Hajjeh et al. (2004)	935	45	24	27
Canada	1999-2004	Laupland et al. (2005)	209	51	22	17
North America	2001-2004	Pfaller et al. (2007a)	2,773	51	22	23
North America	2001-2006	Pfaller et al. (2008a)	1,489	50	24	24
North America	2001-2007	Pfaller et al. (2009b)	11,682	49	21	24
North America	2004-2006	Diekema et al. (2009a)	1,657	52	23	22
North America	2004-2008	Horn et al. (2009)	2,019	46	26	27

Moreover, results from Silva et al. (2011) indicate that infections with both *C. albicans* and *C. glabrata* are of major importance, causing increased tissue damage, since the presence of *C. albicans*

seems to increase the invasiveness of *C. glabrata*. In this way both species are able to occupy similar niches what makes it possible for them to co-exist (Silva et al., 2011).

1.2.2 Candida glabrata

Candida glabrata, together with other *Candida* species, belongs to the class Fungi Imperfecti, the order Moniliales, and the family Cryptococcaceae.

C. glabrata is closely related to the non-pathogenic *Saccharomyces cerevisiae.* It shares a recent common ancestor with several *Saccharomyces* species, and clearly belongs to a clade different from that of other *Candida* species which display particular features such as the recoding of the CUG codon to serine (Roetzer et al., 2011).

It has an optimal growth temperature of 37 °C and, being a nicotinamide adenine dinucleotide (NAD+) auxotroph, its growth is dependent on exogenous supply of NAD+ precursors (Roetzer et al., 2008).

The skeletal component of the cell wall of the majority of fungal pathogens, including *Candida albicans*, and *S. cerevisiae* is based on a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin (a β-(1,4)-linked polymer of N-acetylglucosamine (GlcNAc)). These polymers form hydrogen bonds between adjacent polysaccharide chains to create a tough three-dimensional network of microfibrils. In addition to the glucan and chitin skeleton, *C. albicans* cell wall contains a matrix that mainly comprises glycosylated proteins. In *C. albicans*, the major class of cell wall proteins are glycosylphosphatidylinositol (GPI)-anchor-dependent cell wall proteins (GPI-CWPs), which are attached through a GPI remnant to β-(1,3)-glucan or chitin by a highly branched β-(1,6)-glucan linker (Netea et al., 2008).

The cell wall organization of *C. glabrata* appears similar to that of the closely related nonpathogenic yeast *S. cerevisiae*. However, *C. glabrata* cell wall contains more mannoprotein. These proteins, on the outer layer of the wall of *C. glabrata*, are expected to play key roles in adhesion and biofilm formation and other host-pathogen interactions that mediate fungal virulence (Groot et al., 2008). *C. glabrata* contains covalently bound cell wall proteins (CWPs) that can be divided into two groups, the largest group being glycosylphosphatidylinositol (GPI)-modified proteins that are covalently bound to the wall 1,6-β-glucan whereas the second group is directly bound to 1,3-β-glucan via an ASL (Groot et al., 2008).

1.2.3 Virulence factors

Analyzing *Candida albicans* and *Candida glabrata* virulence factors it is possible to identify some differences and resemblances, what increases the scientific knowledge and consequently makes it possible to evolve clinically, finding efficient therapies and saving more and more infected patients. Several virulence factors were already identified as part of *Candida* species pathogenicity as the morphological behavior on the presence of macrophages, the enzyme production and secretion, the mechanisms of adherence to host cells and non-biological surfaces, the surface cell wall hydrophobicity, the biofilm formation and the resistance to antifungal drugs.

Response to macrophages

Candida albicans resides as a harmless lifelong commensal organism (Mayer et al., 2013), however it is possible to identify some factors that turn it in a dangerous pathogen. One of those virulence factors is its capacity to morphologically switch from yeast cells to pseudohyphae or true hyphae (Whiteway & Oberholzer, 2004). The production of germ tubes results in conversion to a filamentous growth phase or hyphae, also called the mycelial form (Biswas et al., 2007). When on the presence of macrophages, *C. albicans* wild type strain, which is able to make the switch, can kill the macrophages being more effective causing disease. On the other hand, some *C. albicans* mutants (like *cph1/cph1 efg1/efg1* mutant) when interacting with macrophages fail to form filaments and finish suffering phagocytosis and dying (Lo et al., 1997).

In contrast, *Candida glabrata* is not polymorphic, growing only as a blastoconidia (yeast) (Silva et al., 2012). When *C. glabrata* cells are engulfed by host macrophages they have to adapt to the acidic phagosomal environment in order to survive. Inside the macrophages they suffer oxidative stress and they are starved for carbon, so they induce genes involved in gluconeogenesis, β-oxidation, glyoxylate cycle, and transporters for amino acids and acetate (Kaur et al., 2007; Roetzer et al., 2010). *C. glabrata* induces peroxisomes transiently in the process of recycling of internal resources, what plays an important protective role in this condition because the proliferation of peroxisomes in fungal cells contributes to the adjustment of carbon metabolism (Roetzer et al., 2010).

Li et al. (2007), showed that *C. glabrata* even at the lowest infectivity ratio triggers a higher granulocyte macrophage colony stimulating factor response than *C. albicans*, inducing a proinflammatory cytokine response in oral epithelial cells. However, its tissue/cell damaging ability, compared to *C. albicans*, is lower (Li et al., 2007). Schaller et al. (2002) showed the secretion of

epithelial cytokines and chemokines as a potent activator of leukocytes and lymphocytes to the site of mucosal infection. So, as *C. glabrata* cells are able to survive and replicate inside macrophages, this makes it tempting to speculate that luring more macrophages to the site of infection may even be beneficial for the fungus and may constitute part of its immune evasion strategy (Seider et al., 2011). The capability of *C. glabrata* cells to survive, function, and replicate inside the macrophage is due to chromatin remodeling process, that allows energy homeostasis and genes required for its virulence (Rai et al., 2012).

Enzyme production

Another important virulence factor of *Candida albicans* is the secretion of hydrolases. The three most significant extracellular hydrolytic enzymes produced by *C. albicans* are the secreted aspartyl proteinases (Sap), phospholipase B and lipases (Naglik et al., 2003).

SAPs, encoded by a family of ten genes (*SAP1-SAP10*), can be divided into subfamilies based on amino acid sequence homology alignments [*SAP1-SAP3*, *SAP4-SAP6*, *SAP9-SAP10* (*SAP7* and *SAP8* are divergent and are not represented as subfamily members)] (Naglik et al., 2008).

From the three families, it is possible to identify 2 main subfamilies (*SAP1-SAP3* and *SAP4-SAP6*) that are expressed during infections and are believed to be a major virulence factor in *C. albicans* at least for superficial infections (Naglik et al., 2004). Taking in consideration Naglik et al. (2003), there is solid evidence suggesting that Sap9 and Sap10 may not be secreted from the cell and seem to be regulatory proteinases that may play a role in the cell surface integrity, function that differs from the functions of the other Sap. An *in vivo* study addressing the expression and evaluation of *SAP* genes of *C. albicans* was conducted in 137 human subjects with oral and vaginal candidosis, recurring to reverse-transcriptase polymerase chain reaction. According to the results, *SAP2* and *SAP5* are predominant proteinase genes that may be required by *C. albicans* to fulfill basic functions in relation to survival and proliferation in the human host. The other members of the first subfamily presented, *SAP1* and *SAP3*, are more frequently found in association with vaginal infections. The universal expression of *SAP2* during colonization and infection and the preferential expression of *SAP1*, in particular, during active human disease supports the current evidence that the *SAP1-SAP3* subfamily contributes to the pathogenesis and virulence of *C. albicans* at mucosal surfaces (Naglik et al., 2003). Regarding the other *SAP* gene subfamily (*SAP4-SAP6*), the results indicate that *SAP4* and *SAP6* are more frequently expressed during oral and vaginal infections, what indicates that this subfamily is involved in human mucosal disease (Naglik et al., 2003). Sap6, in particular, plays a dominant role during systemic

infections by aiding penetration of tissue and survival of the fungus in phagocytes (Felk et al., 2002). *SAP7* function is completely unknown, however, its expression in both oral and vaginal infections may support a role during human mucosal infections. *SAP8* is expressed in *C. albicans* isolates from active oral and vaginal infections, however the way it contributes to human mucosal infections is still unclear too (Naglik et al., 2003).

An *in vitro* proteinase production assay was performed on several types of *Candida*, including *Candida glabrata*. The results showed that *C. glabrata* produces proteinases (Chakrabarti et al., 1991), however the role of these proteinases is not specified. Some species that are not involved in Sap proteinases secretion may produce enzymes of aspartyl proteinases activity, closely related to *S. cerevisiae* yapsins (Sikora et al., 2011). That's the case for *C. glabrata* as it is closely related to *S. cerevisiae* (Roetzer et al., 2011).

The work of Sikora et al. (2011), showed that all *C. glabrata* strains examined secreted proteinases, due to the detected Yapsin-related aspartyl proteinase genes – *YPS2*, *YPS4*, *YPS6*. They play an important role in the maintenance of cell wall integrity, adherence to host cells, and survival of fungal pathogens in macrophages and in virulence.

Regarding phospholipase production, it has been reported that *C. albicans* has, in total, seven phospholipase genes (*PLA*, *PLB1*, *PLB2*, *PLC1*, *PLC2*, *PLC3* and *PLD1*), but so far only *PLB1*, *PLB2*, *PLC1* and *PLD1* are well characterized, extensively investigated, and also correlated with *C. albicans* pathogenicity. Concerning *PLA*, limited studies were conducted (Samaranayake et al., 2006). The results of the reintroduction of *PLB1* into a *C. albicans* strain to restore and evaluate its virulence *in vivo*, showed that this gene may be responsible for host invasion, degrading the phospholipid constituents of the host cell membranes and allowing fungal hyphae to have access to the cytoplasm (Mukherjee et al., 2001). Regarding *PLB2*, and despite its homology with *PLB1*, any major functional role during colonization and infection in humans can be attributed (Ghannoum, 2000). According to Kunze et al. (2005), the role of *PLC1* is not entirely known but it seems that this is an essential gene, involved in multiple cellular processes and that may in fact act in the nucleus. *PLC2* and *PLC3* are upregulated under filament-inducing conditions but dispensable for filamentation in *C. albicans*, being also not required for escape from macrophages (Knechtle et al., 2005). The function of both *CaPLC2* and *CaPLC3* must be clearly different from *CaPLC1*, as neither of them can compensate for *CaPLC1* loss. The fact that these two genes are unique for *C. albicans* and do not exist in *S. cerevisiae* may suggest a role in adaptation to the human host (Kunze et al., 2005). Hube et al. (2001) performed *in*

vivo and *in vitro* expression studies using Northern blots and reverse transcriptase-PCR and determined that *PLD1* exists in *C. albicans* and expresses higher levels during the yeast to hyphae transition.

A phospholipase assay was performed, on *Candida glabrata* isolates, obtained from blood samples from patients on intensive care units, dialysis units and oncology units, and few of the strains of this species obtained were phospholipase producers (Mohan & Ballal, 2008). This indicates that *C. glabrata* is a phospholipase producer as *C. albicans*.

The *LIP* genes constitute a large gene family that may have evolved to adapt to the permanent association of *Candida albicans* with the human or animal host and, therefore, they may also have important functions during persistence and infection processes (Hube et al., 2000). Fu et al. (1997) transformed *Saccharomyces cerevisiae* with *C. albicans* genomic library and screened for lipolytic activity on egg-yolk agar plates (a traditional screen for phospholipase activity), and two identical clones were obtained and exhibited lipolytic activity. The study results suggested that *C. albicans*, in addition to phospholipases, also had lipases, and by Northern blot analysis, it was shown that expression of the *LIP1* transcript was detected only when *C. albicans* was grown in media containing Tween 80, other Tweens or triglycerides as the sole carbon source (Fu et al., 1997). Strong structural similarities of *LIP2-LIP10* (except *LIP7*) to *LIP1* suggest that like Lip1, they may be as well secreted. In addition, the existence of a family of genes that encode secreted lipases of *C. albicans* may be an indication that different lipase genes are needed during different stages or types of infection (Hube et al., 2000).

The hydrolysis of lipids by the lipases allows *C. albicans* to use fatty acids and/or glycerol as growing substrates transporting hydrolytic products into its cells. The high number of *LIP* genes may provide an advantage to the fungus, allowing it to survive even in the absence of carbohydrates and assisting *C. albicans* in the competition with other microorganisms. Moreover, release of fatty acids may change the pH in the microenvironment (observed decrease from 5.5 to 2.8 during growth in lipid media) allowing the optimal conditions for other fungal proteins like the secreted proteinases (Hube et al., 2000).

In addition, pathogenic microorganisms as *C. albicans* and *C. glabrata* can grow in the host using haemin or haemoglobin as sources of iron. Haemolysins are used by *Candida* species to degrade haemoglobin and facilitate recovery of the elemental iron from host cells. This enables pathogen survival and persistence in the host (Silva et al., 2012).

Adherence to host cells, non-biological surfaces, and surface hydrophobicity

Other important virulence factor is *Candida* adherence to host cells, non-biological surfaces, and its surface hydrophobicity (Luo & Samaranayake, 2002).

As with other pathogenic *Candida* species, the cell wall of *Candida glabrata* is the point of contact between host and fungus. In addition it protects the fungal cell from hostile environments, enabling its adherence to host surfaces and maintaining cell shape (West et al., 2013). Therefore, they thus are excellent targets for development of antifungal drugs and diagnostic tools, being crucial to get more insights into *C. glabrata*'s pathogenesis (Groot et al., 2008).

Cell wall proteins called adhesins are critical to mediate adhesion of *C. albicans* to various substrates, like abiotic surfaces, and other yeast cells, microorganisms or host cells. Among many adhesins, members of the agglutinin-like sequence (Als) family of glycoproteins are particularly active in cell aggregation, adhesion to endothelia and epithelia, adherence to a broad variety of host substrates and consequent infection of those host surfaces, formation of biofilms and pathogenesis in mouse models (Garcia et al., 2011; Phan et al., 2007). However, Als3 and Als1 are the only members of this family that have a significant invasive function being capable of inducing epithelial cell endocytosis by itself. Als6, Als7 and Als9 do not appear to mediate significant endothelial cell endocytosis, and Als2 and Als4 have not yet been tested (Phan et al., 2007). The study of the proteins that may have a function in adherence led to the identification of a hypha-specific surface protein (Hwp1). According to the results, the presence of Hwp1 might enable *C. albicans* to form tight attachments to the oral mucosa through TGase-catalyzed cross-linking between Hwp1 and epithelial cell proteins, which is important in the pathogenesis of candidiasis (Staab et al.,1999). Morphology-independent proteins can also contribute to adhesion. These include GPI-linked proteins (Eap1, Iff4 and Ecm33), non-covalent wall-associated proteins (Mp65, a putative β - glucanase, and Phr1, a β -1,3 glucanosyl transferase), cell-surface associated proteases (Sap9 and Sap10) and the integrin-like surface protein Int1 (Mayer et al., 2013).

On *C. glabrata* cell wall, there is one group called the *EPA* (epithelial adhesin) genes that are not present in the *Saccharomyces cerevisiae* genome. This subtelomeric gene family encodes adhesion proteins. Approximately 67 genes encoding adhesion-like glycosylphosphatidylinositol (GPI) - anchored proteins reside within the *C. glabrata* genome, and at least 17 or 23 (depending on the background) of these proteins can be allocated to the Epa family (Groot et al., 2008; Kaur et al., 2005). According to Cormack et al. (1999), Epa1 is responsible for 95% of the adherence to human epithelial cells *in vitro*. The presence of angiotensin-converting enzyme – 2 (Ace2) in the *C. glabrata* cell wall is also important

to mention. *C. glabrata* Ace2 is a homologue of the *S. cerevisiae* transcription factor Ace2 (Stead et al., 2009). According to data from a murine model of candidiasis, inactivation of *Cg*Ace2 results in a hypervirulent strain whose infection results in a fatal systemic disease on 100% of the infected mice (Kamran et al., 2004).

Adherence to non-biological surfaces is essentially important in a clinical environment, where it is important to make sure that all indwelling devices used on patients are not infected by *C. albicans* or *C. glabrata* and do not contribute to increase patients' illness. These concern is due to the fact that these devices represent a potential niche for adhesion and further formation of biofilm-associated infections (Kucharíková et al., 2014).

The surface hydrophobicity is another virulence factor for *Candida albicans*. According to the results of an *ex vivo* assay in mouse tissue, hydrophobic yeast cells are able to bind to host tissues diffusely and abundantly (Hazen et al., 1991). These results of Hazen et al. (1990) indicated in that *C. albicans* hydrophobicity was due to changes in the external surface protein exposure and that protein mannosylation would influence exposure of hydrophobic surface proteins. Hydrophobic cells are more resistant than hydrophilic cells to phagocytic killing (Antley & Hazen, 1988). Also for *C. glabrata*, Epa6 mediates strong hydrophobic interactions, as a basis for biofilm formation on abiotic surfaces (El-Kirat-Chatel et al., 2015).

Biofilm formation

A biofilm can be defined as an aggregate of microbial cells adherent to a living or nonliving surface, embedded within a matrix of extracellular polymeric substances (EPS) of microbial origin (Hall-Stoodley et al., 2012). It confers an advantage for all microorganisms being important for their survival as commensals and pathogens of humans. It allows them to evade host immune mechanisms and to resist both treatment with antifungals and competitive pressure from other microorganisms. In addition, biofilm formation may allow the species to be better adapted to colonization of tissues and indwelling devices (Silva et al., 2009).

Candida albicans cell wall proteins called adhesins are critical for biofilm formation. Among many adhesins, the members of the agglutinin-like sequence (Als) family of glycoproteins are particularly active in formation of biofilms and pathogenesis in mouse models (Garcia et al., 2011). Moreover, phospholipase B genes have temporal differences in gene expression levels in biofilms, what was observed to be model-dependent. *PLB1* was downregulated in biofilm growth in microtiter plates (MTP) and in the *in vivo* and reconstituted human epithelium (RHE) models, however not in those

grown on silicone disks in a continuous flow system (CDC reactor). *PLB2*, was also underexpressed in biofilms grown in the MTP and in the *in vivo* and RHE models (up to 12 hours), and was upregulated in biofilms grown in the CDC reactor and in the RHE model (after 24 hours and 48 hours) (Nailis et al., 2010).

Using a clinically relevant model for subcutaneous catheter-related infection on rat, *in vivo* biofilms were compared with 6-day-old *in vitro* biofilms formed on the same devices placed in a 24-well plate and submerged in RPMI 1640 medium. A subset of genes – *EPA1*, *EPA3*, *EPA6* and *AWP1-AWP7* was chosen. Comparing the *in vivo* model system results with planktonic cells incubated in RPMI 1640 medium for 6 days with continuous shaking, it is possible to see that the studied genes are upregulated on the *in vivo* conditions. In addition, comparing the results from 6-day-old *in vitro* biofilms with planktonically grown cells, the genes of the *EPA* gene family chosen to study (*EPA1*, *EPA3* and *EPA6*) were not significantly differentially expressed, whereas non-*EPA* adhesion genes, such as *AWP3*, *AWP6* and *AWP7*, were up-regulated. Comparing the expression levels in 6-day-old *in vivo* biofilm with 6-day-old *in vitro* biofilm, it was verified the up-regulation of *EPA3* and *EPA6* for the *in vivo* assay, whereas the expression of *EPA1* was not different from that under *in vitro* conditions. These different results point to the fact that the infection process strongly depends on the environment. *EPA3* expression results (at least 4-fold higher *in vivo* than *in vitro*) might be an indication that it is one of the most important genes responsible for *in vivo* biofilm development in a subcutaneous model. Regarding the *AWP* gene family, all genes shown to be expressed however increased expression of *AWP2*, *AWP3* and *AWP5* was observed in mature *in vivo Candida glabrata* biofilms (Kucharíková et al., 2014). Epa6, as already referred, mediates adherence to epithelial cells (Castaño et al., 2005), being an adhesin required for biofilm formation in *C. glabrata*. *EPA6* and its close paralogue *EPA7* are located in subtelomeric regions and their transcription is regulated by Sir4p and Rif1p, two proteins involved in subtelomeric silencing. Biofilm growth conditions induce the transcription of *EPA6* and *EPA7*: this is dependent on the presence of an intact subtelomeric silencing machinery and is independent of the Mpk1p signalling pathway. Finally, the kinase Yak1p is required for expression of both adhesin genes and acts through a subtelomeric silencing machinery-dependent pathway (Iraqui et al., 2005).

The ability of *Candida* to form biofilm is an important feature that promotes both infection and persistence in the host. Biofilm activity is significant since high activity might be associated with enhanced expression of putative virulence factors. For *C. glabrata*, it was shown that their biofilms had lowest metabolic activity but higher number of cultivable cells per unit area when compared with other species studied – *Candida tropicalis* and *Candida parapsilosis* (Silva et al., 2010). In addition, the

biofilm matrices from *C. glabrata* have higher amounts of both proteins and carbohydrates than those species (Silva et al., 2009). Moreover, *C. glabrata* was unable to generate filamentous forms unlike the other *Candida* species tested (Silva et al., 2010). Thein et al. (2007) reported that after 48 hours, the biofilms of aerobically grown *C. glabrata* generally revealed a multilayer biofilm structure packed with blastospores devoid of either pseudohyphae or hyphae. Under anaerobic/static conditions, *C. glabrata* forms non-contiguous microcolonies of blastospores (Thein et al., 2007).

Tolerance to azole antifungals

One important virulence factor of *Candida glabrata* is its tolerance to azole antifungal drugs. It has been demonstrated that resistance to fluconazole is associated with an increased expression of the ATP binding cassette (ABC) transporters *CgCDR1* and *CgPDH1*, which appeared to occur after patient exposure to this drug (Bennett et al., 2004). Another study demonstrates that some *C. glabrata* isolates can acquire decreased susceptibility to azole drugs, during exposure to azole compounds (Panackal et al., 2006).

For more information about other antifungal drugs see the section *Antifungal classification and resistance*.

Melanin-like pigment

Another *Candida glabrata* virulence factor is the production of a melanin-like pigment, since *C. glabrata* cells have a high rate of survival when treated with hydrogen peroxide (Brunke et al., 2010). A decreased neutrophil-mediated damage was seen in the presence of this melanin-like pigment and increased ability to cause epithelial damage. However *C. glabrata* pigment is formed as a by-product of tryptophan catabolism via the Ehrlich pathway. The gene *ARO8* is transcriptionally induced in the medium containing tryptophan as the sole nitrogen source, and its product Aro8 can generate indole pyruvate from tryptophan. This intermediate is then either catabolized further to indole acetaldehyde by *ARO10*, or is secreted into the supernatant. Once outside the yeast cell, the intermediate spontaneously reacts to form the pigment only if oxygen is present. As the pigmented cell cause increased damage *in vitro*, expression of the pigment *in vivo* would explain in part the pathogenic potential of *C. glabrata*. However, the pigment production by *C. glabrata* is limited to very specific conditions so it is a rather unlikely event in any relevant *in vivo* scenario of *C. glabrata* infections (Brunke et al., 2010).

1.2.4 Antifungal classification and resistance

Nowadays, the therapeutic options for invasive fungal infections are limited to three most important classes of antifungals: polyenes, azoles and echinocandins (Figure 2).

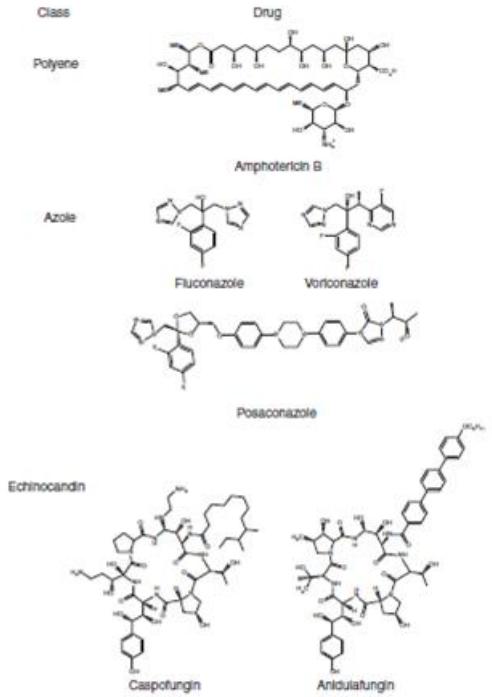


Figure 2 - Representation of different classes and antifungal drugs in use nowadays (Roemer & Krysan, 2014).

The limited options are firstly due the fact that, unlike antimicrobials targeting bacteria, fungal pathogens are more closely related to the host. As many fundamental biochemical and cell biological processes are conserved from fungi to humans, many small molecules that are toxic to yeast are also toxic to humans. For this reason the antifungal drug has to target structures unique to fungi as the three antifungals classes do (Roemer & Krysan, 2014). Secondly, the development of a new antifungal agent requires many economic resources as much as time for finding the new compound and to test its security and efficacy. Then, years are required to proceed to clinical trials and to turn the new antifungal into an approved drug.

Antifungal resistance can be divided into *in vitro* and clinical resistance. The first, *in vitro* resistance is a laboratory measurement incorporating clinical experience and set against the range of susceptibility results for particular drugs and fungal species. It can be divided into primary/intrinsic resistance in which the fungus is resistant prior to drug exposure, or secondary/acquired resistance where the fungus resistance develops in response to drug exposure. Clinical resistance is defined as the

persistence or progression of the fungal infection and it occurs despite the intervention of the adequate antifungal therapy, in the appropriate dose and by the most efficient administration route (Rogers, 2006). Antifungal resistance results of the combination of both *in vitro* and clinical resistance. This means that resistance is present if isolates are not inhibited by an antifungal drug administrated at normal doses and normal dosage schedules. In addition to this definition, MIC values can be a sign of antifungal resistance (Pfaller, 2012).

Polyenes

All the polyenes interact with sterols, change the membrane permeability of eukaryotic cells and lead to cell lysis. Taking in consideration the classification of polyene antibiotics according to its chemical structure and biological effects by Kotler-Brajtburg et al. (1979), it is possible to distinguish between two groups. The first group includes those which caused little or no K^+ leakage or growth inhibition without cell killing or lysis and the second group includes those producing significant K^+ leakage or growth inhibition at much lower concentrations than concentrations required for cell lysis or death (Kotler-Brajtburg et al., 1979).

Amphotericin B, the most important polyene, acts by binding to sterols in the fungal cell wall and altering membrane permeability, thereby allowing the leakage of cytoplasmic components (Khoo et al., 1994) as K^+ and Mg^{2+}. These losses, along with a subsequent influx of protons into the fungal cell, cause acidification of the fungal interior with precipitation of the cytoplasm and ultimate cell death (Hamill, 2013).

This molecule is delivered by intravenous infusion and is the agent with the broadest antifungal spectrum. However, it has some important side effects as fever and chills, rigors, arthralgias, nausea vomiting and headaches. In addition, it presents a substantial incidence of renal toxicity. For this reason, amphotericin B lipid formulations were developed to reduce toxicity and provide a safer alternative than conventional amphotericin B (Hamill, 2013). The disadvantage of such formulations is the quite expensive price and the fact that are available only in some regions (Roemer & Krysan, 2014).

The targets of polyenes are plasma membrane sterols. However, the mechanisms of resistance to amphotericin B are poorly understood, but a decrease or lack of ergosterol content in the fungal cell membrane has been associated with resistance without affecting cell viability. Defects in the ERG3 gene have been reported to lead to an accumulation of other sterols instead of ergosterol (Pemán et al., 2009).

It was observed a decrease in *Candida glabrata* susceptibility to amphotericin B in some regions like Europe where 4.4% of the isolates had MIC values in excess of 2 μg/ml (Pfaller et al., 2004). This means an increase of resistance so higher doses of amphotericin B may be required for optimal treatment of infections with this particular pathogen (Nguyen et al., 1998).

Azoles

Azole antifungal drugs consist of a five-membered ring containing two or three nitrogens, classified as imidazoles (as ketoconazole, miconazole and clotrimazole) or triazoles (as itraconazole and fluconazole), respectively. The use of imidazoles is limited to treatment of superficial mycoses (with the exception of ketoconazole), whereas triazole drugs have a broader range of applications in the treatment of both superficial and systemic fungal infections (Sheehan et al., 1999). This class of antifungal agents blocks ergosterol biosynthesis by inhibition of lanosterol 14α-demethylase, leading to a diminished ergosterol production. Therefore cells lack the ability to build and renew sterols in cell membranes, changing the membrane fluidity and function of vital processes such as: signaling, transport, exocytosis and endocytosis (Rodrigues et al., 2013).

Azoles are extremely well tolerated, although they interfere with the metabolism of a number of other drugs owing to their ability to inhibit cytochrome P450. In general, fluconazole has broad activity against clinically relevant yeast including *Candida* species and *Cryptococcus*. Because amphotericin B and 5-flucytosine are not available in many resource-limited regions, fluconazole is widely used to treat cryptococcal meningitis despite the fact that it is less effective. Moreover, many isolates of *Candida glabrata* and *Candida krusei*, however, are intrinsically less susceptible (Roemer & Krysan, 2014).

Four major mechanisms of resistance to azoles have been described in *Candida* species: (i) decrease of intracellular drug concentrations, (ii) target site alteration, (iii) overexpression of the target enzyme and (iv) development of a bypass pathway. *Candida* cells can develop a decreased drug concentration by upregulation of active efflux pumps. According to a multiple efflux mechanism study (Albertson et al., 1996), resistance to fluconazole can be acquired by mutations causing an increased expression of *CDR1*, while other mutations causing increased *CDR1* expression are associated with multiazole resistance. In a different study, a new ABC transporter closely related to Cdr1 that was named Cdr2, was cloned. The protein Cdr2p could confer resistance to azole antifungal agents, other antifungals (terbinafine, amorolfine) and to a variety of metabolic inhibitors (Sanglard et al., 1997). Another family of transporters is the Mdr family where Mdr1 appears to be the major facilitator (White,

1997). For *Candida albicans*, the expression of increased mRNA levels of MDR1 and CDR are associated with increased resistance (White, 1997).

A second mechanism of resistance in *Candida* species is the acquisition of point mutations in the gene encoding for the target of the azoles (*ERG11*). This results in an altered target that has a reduced affinity or is incapable to bind to the azole drugs (Pfaller, 2012).

Candida can also overexpress or upregulate the Erg11 target enzyme, leading to a deficient binding of targets with azole drugs, or to an increased quantity of those drugs. However, minimal upregulation of target enzymes has been observed to date, and this mechanism does not appear to be a major cause of azole resistance in *Candida* at this time (Pfaller, 2012). The antifungal agent is, therefore, overwhelmed and routine therapeutic concentrations can no longer effectively inhibit ergosterol synthesis. Target enzyme up-regulation can be achieved through gene amplification, increased transcription rate, or decreased degradation of the gene product (Kanafani & Perfect, 2008).

The last mechanism of azole resistance in *Candida* species is associated with the development of bypass pathways, which difficult the disruptive action on the membrane by the azole drugs. This has been linked with mutations in *ERG3* in certain resistant strains of *Candida* (Pfaller, 2012). After fluconazole treatment, the predominant ergosterol in the fungal membrane is 14α-methyl-3,6-diol. Mutations in the *ERG3* gene preventing the accumulation of 14α-methyl-3,6-diol, allow the formation of the precursor 14α-methylfecosterol, which allows *Candida* to grow even in the presence of the drug (Kelly et al., 1997). Therefore, replacement of ergosterol with the latter product leads to functional membranes and abolishes the action of azoles on the ergosterol biosynthetic pathway (Kanafani & Perfect, 2008).

According to *Artemis Disk Global Antifungal Surveillance Study* data, *C. glabrata* strains from North America (21.1% from all the *Candida spp.* in this region against 11.3% in Europe), exhibit higher rates of azole resistance than strains from Europe (19.5% for fluconazole in North America compared to 16.3% in Europe) (Pfaller et al., 2010). In general, health care unit patients infected with this pathogen have a higher risk of receiving an inadequate therapy, in comparison with those infected by fungal pathogens susceptible to azoles (Klevay et al., 2009).

For this reasons, the common use of fluconazole as initial therapy to treat suspected candidemia, may not be advisable due to *C. glabrata*'s reduced susceptibility to azoles (Klevay et al., 2009). However it is better to treat *C. glabrata* infections with an amphotericin B based drug, rather than fluconazole (Nguyen et al., 1996).

Echinocandins

The echinocandins are a class of drugs that target the cell wall by inhibiting β-1,3-D-glucan synthesis. The drug induces the formation of a defective cell wall, leading to cell rupture in yeasts and aberrant hyphal growth in molds (Kanafani & Perfect, 2008).

They have broad fungicidal activity against *Candida* species and have emerged as an important therapeutic option for candidiasis. For many of the most common invasive fungal infections, the better tolerated azoles and echinocandins have emerged as first-line agents (Roemer & Krysan, 2014). However, in comparison with fluconazole, echinocandins proved to be very powerful drugs, eliminating *Candida glabrata* biofilm-associated infections. Micafungin, caspofungin and anidulafungin significantly decreased mature *C. glabrata* biofilms developed subcutaneously after 7 days of treatment. The high efficiency of echinocandins against biofilms might result from their fungicidal nature, although increasing use of echinocandins in the therapy of patients leads to rising numbers of *C. glabrata* resistant strains (Kucharíková et al., 2014).

The mechanisms of echinocandin resistance are still being investigated (Kanafani & Perfect, 2008) however, an assay with *Candida albicans* strains allowed confident detection of mutations in *FKS1* gene conferring reduced susceptibility to caspofungin. The mutations are associated with alterations of serine 645 of Fks1p (Balashov et al., 2006).

It was demonstrated for a minority of *C. albicans* strains and specifically for caspofungin that growth occurs at concentrations well above the MIC. This paradoxical effect appears to be less common with other *Candida* species (Stevens et al., 2004). It was shown that caspofungin has an effect on cell wall hexoses, including glucans and chitin. The glucan content is depressed by the drug and the chitin content is upregulated. The paradoxical effect seems to be due to an increase in the chitin content on the cell wall of *Candida* isolates that allow them to survive in the presence of high concentrations of caspofungin. According to the same data, caspofungin also inhibits synthesis of β-1,6-glucan and β-1,3-glucan (Stevens et al., 2006).

There are several studies published about cases of susceptibility reduction of *C. glabrata* isolates to echinocandins like caspofungin. Concerning *C. glabrata* isolates from a patient in an intensive care unit, recovered from gall drainages and urine, resistance to caspofungin was found (Krogh-Madsen et al., 2006). Another study of caspofungin effect on *C. glabrata* clinical isolates confirmed the role of *C. glabrata FKS2* mutation on the reduction of echinocandin susceptibility (Katiyar et al., 2006).

Other antifungals

In another antifungals class it is possible to identify flucytosine, a base pyrimidine analogue that inhibits cellular DNA and RNA synthesis. However, organisms have already developed acquired resistance to this antifungal drug that resulted from defects in its metabolism through enzymatic mutations (Pemán et al., 2009).

According to Pfaller & Diekema (2007), it is apparent that no class of antifungal agent is immune to the development of resistance. In addition, mortality attributable to invasive candidiasis remains high largely due to delays in the administration of appropriate antifungal therapy (Pfaller & Diekema, 2007). Because of the increasing failure of treatment, new targets for novel therapeutic approaches are required (Kucharíková et al., 2014) to assure the following generations to have a safe and efficient therapy capable of assuring humans health.

2. CHAPTER II

Materials and Methods

2.1 Strains

The *Candida glabrata* strains used in this work were: *C. glabrata* (ATCC 2001); a *his3Δ trp1Δ leu1Δ* strain (ATCC 2001 background); the BG2 strain (that contains Epa1p, which is a member of the GPI protein family) (Schwarzmüller et al., 2014); a clinical isolate from the Leuven hospital and 619 strains from the recently published deletion collection (Schwarzmüller et al., 2014).

The *Saccharomyces cerevisiae* strains used were W303-1A (possesses a ybp1-1 mutation (I7L, F328V, K343E, N571D) which abolishes Ybp1p function, increasing sensitivity to oxidative stress); S288C (strain used in the systematic sequencing project and that does not form pseudohyphae) and Σ1278b (genome is closely related to S288c, and shares some other genomic regions with W303).

The *Candida albicans* strain used was SC5314 (wild type strain).

2.2 Secretion Analysis by Strain Spotting

To study the secretion of cells of different species (*Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae*), several strains were spotted on two different medium plates: SC with 2% agar plus 5% glucose and SC with 2% agar plus 5% glucose plus 5% fetal bovine serum (FBS) (Gibco®) (5 % FBS had the most significant effect on a *C. glabrata* growth curve with different concentrations of serum). *C. glabrata* strains were spotted (including the wild type, a clinical isolate, a *his3Δ trp1Δ leu1Δ* strain (ATCC 2001 background) and BG2). This way, wild type ATCC 2001 was used as a control and, as several other strains were spotted, it was possible to verify if there was a strain that produced a bigger, lesser or no secretion. This assay was based on the comparison between the secretion that different strains would produce on the presence and absence of FBS. Therefore, *S. cerevisiae*, as a close related microorganism of *C. glabrata*, and *C. albicans*, the most common and pathogenic *Candida* sp., were included. The *C. glabrata* plates were incubated at 37 °C and the *S. cerevisiae* and *C. albicans* plates were incubated at 30 °C and checked each 24 h, during 6 days.

2.3 SDS-PAGE

2.3.1 Sample preparation

Cultures of 24h of ATCC 2001 in synthetic complete (SC) medium (0.77 g/l complete supplement mixture (CSM) (formedium); 1.7 g/l yeast nitrogen base without amino acids (Remel) and

5 g/l ammonium sulfate (Sigma Aldrich); pH 6.5) plus 2% glucose and in SC plus 2% glucose and 5% FBS were centrifuged at 500 rpm during 10 min at 4 °C. Then the supernatant of each culture was kept, removing the pelleted cells. This step was repeated, with increased rounds per minute (rpm). After the last centrifugation step, each culture was checked for the absence of cells by observation under the microscope.

Then, as cells were absent, a centrifugation at 14000 rpm (high speed centrifugation) at 4 °C, during 3 min was made to pellet the proteins left.

Afterwards, the pellets were washed with Tris-EDTA (TE) buffer (100 mM/L Tris(pH7.4) + 10 mM/L EDTA(pH8.0)). Therefore 1 ml of TE buffer was added in the samples incubated on ice for 5 min. A high speed centrifugation at 4 °C, during 3 min was made and the supernatant was discarded in the end. Again 1 ml of TE buffer was added to assure that the wash was efficient. After another centrifugation step, the pellet was resuspended in 500 µl buffer solution (4% SDS, 1% DTT and 100 mM Tris (MW=121.12 g/mol); HCl till pH = 7.6).

2.3.2 SDS-PAGE

A solution with 40 µl of each sample and 10 µl loading dye (30% v/v β-mercapto-ethanol, 250 mM Tris pH=8, 10% SDS, 0.5% bromofenol blue, 50% glycerol) was performed. Sodium dodecyl sulfate (SDS) dissolves cells membranes and denaturates the proteins covering them with negative charges (because of the sulfate group). This way, when on an electric field, the proteins are able to migrate towards the positive pole. Glycerol has a high density which assures the loading of the samples in the gel. Bromophenol blue is a dye to see the progression of the electrophoresis. Next, the samples were heated at 65 °C during 5 min and this was centrifugated at 3000 rpm during 4 min. Then, 10 µl of the samples and a molecular weight ladder (SeeBlue® Plus2 Pre-stained Protein Standard) were loaded in the gel. The gel was run by an electric current of 100 V for 10 min and subsequently 150 V for 75 min.

2.3.3 Gel staining

The gel was removed from the cassette and rinsed 3 times with 100 ml Milli-Q water during 5 min. To stain the gel, 20 ml Simply Blue™ Safe Stain (LifeTechnologies) was added to cover the gel and this was incubated for at least 1 h at room temperature while gently shaking. Subsequently, 100 ml Milli-Q water was added to destain the gel, during at least one hour.

2.4 Extraction of Proteins via SDS for LC MSMS

After carrying out the section *SDS Page – Sample Preparation*, protein concentrations were determined using the nanodrop 2000.

2.4.1 SDS removal and digest

To remove the remaining SDS from the protein samples, 20 µg protein from each sample was mixed with 400 µl Urea buffer (UA) (8 M urea in 0.1 M Tris/HCl (pH 8.5)). The samples were added on Microcon YM-30 filters (Millipore, Billerica, MA, USA) and centrifuged (13 000 rpm; 20 min). The flow-through was discarded and the samples were washed twice with UA-buffer (Kraft-Terry & Gendelman, 2012). Then, 200 µl UA-buffer containing 20 mM 1% dithiothreitol (DTT) was added to the filter. After 15 min incubation at room temperature, the samples were centrifuged again (14000 rpm; 20 min). 100 µl UA-buffer containing 0.05 M of iodoacetamide (IAA) was added to the filters, incubated for 30 min and centrifuged again. Then, 200 µl UB-buffer (8 M urea in 0.1 M Tris/HCl (pH 8.0)) was added to the filters, followed by centrifugation at 14 000 rpm during 20 min. This step was repeated twice. Again 40 µl UB-buffer was added, containing Lys-C (enzyme-protein ratio of 1:50) and this was incubated overnight at room temperature. Afterwards, 120 µl of 40 mM ammonium bicarbonate (ABC) containing trypsin (enzyme-protein ratio 1:100) was added to the filters, followed by incubation during 4 h at 37 °C (Antunes et al., 2014).

After digestion, the filters were centrifuged at 14 000 rpm during 20 min but this time the flow-through was kept. In addition, 50 µl of 0.5 M NaCl was added to the filters following centrifugation. The flow-through was collected (Antunes et al., 2014).

2.4.2 Salt removal

Pierce C18 Spin Columns (Thermo Scientific) were used to remove the salts from the samples. The digested samples were acidified with a buffer containing 2% trifluoroacetic acid (TFA) and 20% acetonitrile (ACN) (sample: buffer ratio 3:1) resulting in a final concentration of 0.5% TFA and 5% ACN. The C18 columns were activated and equilibrated by adding 200 µl of 50% ACN and 200 µl 0.5% TFA in 5% ACN respectively, followed by centrifugation at 1,500 *g* for 1 min after each step. The samples were added to columns and centrifuged at 1,500 *g* for 1 min. This step was repeated once to maximize the sample binding to the column. The columns were washed with 200 µl of 0.5% TFA in 5% ACN, and centrifuged at 1,500 x g for 1 min. This step was repeated once to remove the maximum amount of salt contaminants. The samples were eluted from the column by applying twice 25 µl of 70% ACN and

centrifuged at 1,500 *g* for 1 min. Samples were dried in a vacuum operator until they were dry and the peptides were dissolved in 10 µl of 0.1% FA and 5% CAN (Antunes et al., 2014).

Then the stock TFA solution was diluted 10 times with the protein sample. The sample was stored in the freezer and then transfer to be analysed by mass spectrometry at SyBioMa (Katholieke Universiteit Leuven Association, Belgium).

2.5 Screening for Defective Secretion

A large-scale *Candida glabrata* deletion library consisting of 619 strains was used to screen for defective secretion (Schwarzmüller et al., 2014). The strains were grown in 200 µl of SC medium with 2 % agar in a 96-well format overnight at 37 °C while shaking. Subsequently 96-well plates were filled with 200 µl SC agar medium plus 2% D-glucose and 5% FBS. The overnight culture was pinned on the agar 96-well plates, incubated for two days at 37 °C and checked for secretion.

2.6 Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) value corresponds to the lowest concentration of an antimicrobial agent that causes a specified visible reduction in the growth in a broth dilution susceptibility test. The CSLI standard method used allows setting levels of microbial resistance to an antimicrobial agent after a specific incubation time (Rex et al., 2008).

The MIC was determined in 96-well plates. An amount of 80 µl of SC media plus 2% glucose or SC media plus 2% glucose plus 5% FBS was added to each well together with 20 µl of a 10 times concentrated antifungal solution. The fluconazole concentrations used, as documented on MIC guidelines (Rex et al., 2008), was a serial twofold dilutions indexed to the base 2 (final concentrations: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 µg/ml). Those values already include the MIC value previously obtained for *Candida glabrata* cells treated with fluconazole: 8 µg/mL (Rex et al., 2008). The highest and lowest concentrations were chosen due to the unpredictable MIC value for *C. glabrata* cells cultured on FBS when treated with fluconazole and because of the drug concentration ranges used previously (Rex et al., 2008). The wild type *C. glabrata* (ATCC 2001) was counted in a Neubauer counting chamber and diluted in H_2O to 5000 cells/ml. Then, 100 µl of cell suspension (in the indicated medium) was added to each well. The plates were incubated at 37 °C during 24 h and the

cell growth was evaluated visually, according to the acceptable time of MIC reading for this specific antifungal agent (Rex et al., 2008).

2.7 Spot Assay – Congo Red and Calcofluor White

Synthetic Complete (SC) medium with 2% glucose and 1.5% agar was prepared. Each condition was tested in the presence and in the absence of 5% FBS. In a first condition 100 µg/ml or 200 µg/ml calcofluor white (CW) was added. Secondly, 1 mg/ml or 3 mg/ml Congo red (CR) was tested. A tenfold dilution series of *Candida glabrata* (ATCC 2001) was spotted on these plates (start OD_{600} of 0.5). This was incubated at 37 °C for 24 h or 48 h.

2.8 Adhesion Assay

A Yeast Extract Peptone Dextrose (YPD) pre-culture of wild type *Candida glabrata* ATCC 2001 was grown at 37 °C while shaking during 24 h. The culture was washed twice in Phosphate-Buffered Saline (PBS 1x: 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM K_2HPO_4 in 1L H_2O with pH 7.4) and resuspended in 1 ml PBS (1X). A 1/1000 dilution in PBS was prepared to determine the number of cells per ml using a Neubauer counting chamber. The cell suspension was diluted to $1x10^7$ cells per mL in the indicated medium. Subsequently 10^6 cells were added to each well of a 96-well plate. The wells from the edges were not used because of vaporization and for each condition, at least one well was filled only with medium (blank). The 96-well plates were incubated at 37 °C during exactly 90 min. The wells were washed two times with 150 µl of PBS (1x) to assure that only the adherent cells were remaining in the wells. After this step, the cells were treated with a XTT menadione solution in the adhesion assay.

Adhesion assay quantification: 100 µl of a solution of 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) (XTT) (1 mg/ml) and menadione (1µM) was added to each washed well and this was incubated for 1 h in the dark at 37 °C without shaking. XTT is converted to an orange coloured formazan product by mitochondrial enzymes in the adherent *Candida* cells. Menadione is an artificial electron transporter that is added to drastically induce the reduction to formazan derivates (catalyst). In the absence of menadione, the XTT reduction would require an incubation of almost 24 h.

3. CHAPTER III

Results and Discussion

3.1 Evaluation of the Secretion Produced by *C. albicans* and *S. cerevisiae*

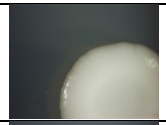
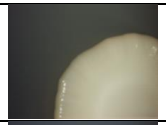
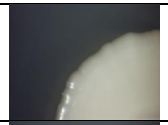
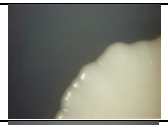
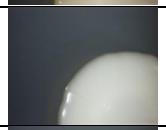
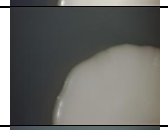
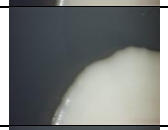
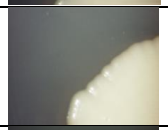
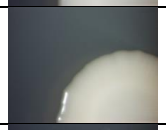
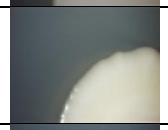
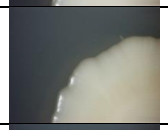
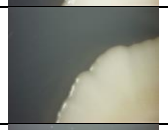
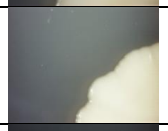
To verify if the secretion observed in *Candida glabrata* cultures (Wild Type ATCC 2001) is also present in *Candida albicans* (SC5314) and *Saccharomyces cerevisiae* strains (W303-1A, S288C W303-5A, Sigma1278b), different strains were spotted on plates containing minimal medium containing glucose and 5% FBS (Tables 2 and 3). Different *C. glabrata* strains (Wild Type ATCC 2001, Clinical Isolate, HTL, BG2) were used as a control.

From these results it is possible to conclude that all the strains, and not only *C. glabrata*, show secretion. The secretion was only present on the strains cultured with FBS (Table 3).

From all the *C. glabrata* strains tested, HTL (*his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT) (Schwarzmüller et al., 2014) seems to be the one that secretes first (immediately at 24 hours) and in higher proportion (see Table 3).

From all *S. cerevisiae* strains tested, W303-1A – that presents increased peroxide sensitivity (Veal et al., 2003) - and S288C Wa3-52 seem to be the ones that secrete first (at 48 hours) however W303-1A presents a higher secretion than the second strain (see Table 3) and that it because this last *S. cerevisiae* strain is more susceptible to oxidative stress *in vitro* and has a higher pronounced response to it than *C. glabrata* strains, that are more resistant (Roetzer et al., 2008). The secretion is faster for *S. cerevisiae* strains in comparison with *C. glabrata* and *C. albicans* strains.

Table 2 - Representation of the evolution of the secretion for different species and strains each 24 hours till 144 hours, cultured on minimal medium with glucose (SCD)

Species	**Strains**	**Temporal periods studied**					
		24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
C. glabrata	**Wild Type ATCC 2001**						
	Clinical Isolate						
	HTL						
	BG2						
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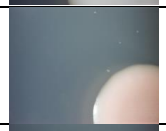
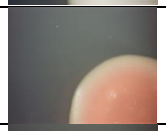
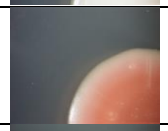
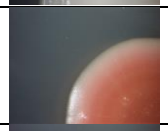
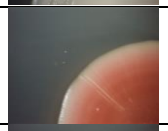
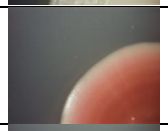
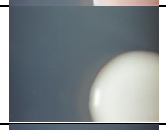
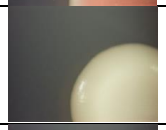
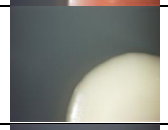
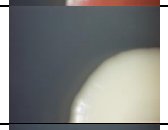
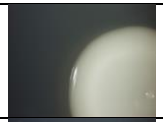
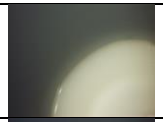
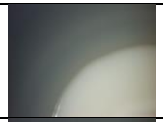
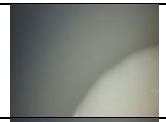
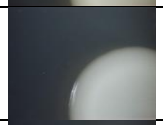
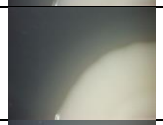
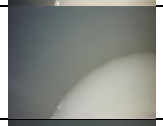
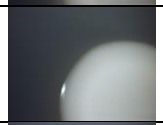
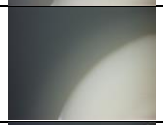
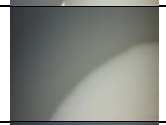
S. cerevisiae	**W303-1A**						
	S288C Wa3-52						
	Sigma1278b						

Table 3 - Representation of the evolution of the secretion for different species and strains each 24 hours till 144 hours, cultured on minimal medium with glucose (SCD) and FBS

Species	**Strains**	**Temporal periods studied**					
		24 hours	48 hours	72 hours	96 hours	120 hours	144 hours
C. glabrata	**Wild Type ATCC 2001**						
	Clinical Isolate						
	HTL						
	BG2						
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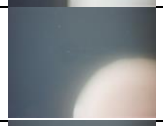
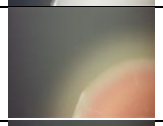
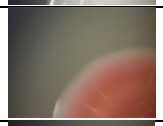
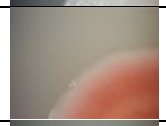
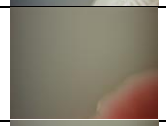
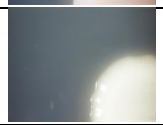
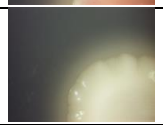
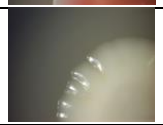
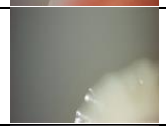
S. cerevisiae	**W303-1A**						
	S288C Wa3-52						
	Sigma1278b						

Since *C. glabrata* and *S. cerevisiae* are closely related (Roetzer et al., 2011), both species were grown in presence as well as in absence of FBS. The resulting samples were analysed by SDS-PAGE and SimplyBlue™ SafeStain (Figure 3). According to these SDS-PAGE results, *S. cerevisiae* also secretes proteins in the presence of FBS (Figure 3 - lane 10). However, this pattern is much less dense than *C. glabrata* (Figure 3 - lane 2) what may be due to the abnormal high presence of proteins on the remaining supernatant on the *S. cerevisiae* Σ1278b culture in SCD plus FBS (Figure 3 – lane 11). Taking in consideration that most of the proteins secreted are on the *S. cerevisiae* Σ1278b culture in SCD plus FBS remainant supernatant, this result may be according to the previously obtained on the secretion of several strains from *C. glabrata* and *S. cerevisiae*, when it was concluded that *S. cerevisiae* strains are more susceptible to oxidative stress than *C. glabrata* strains. This indicates that there are some differences between the two species.

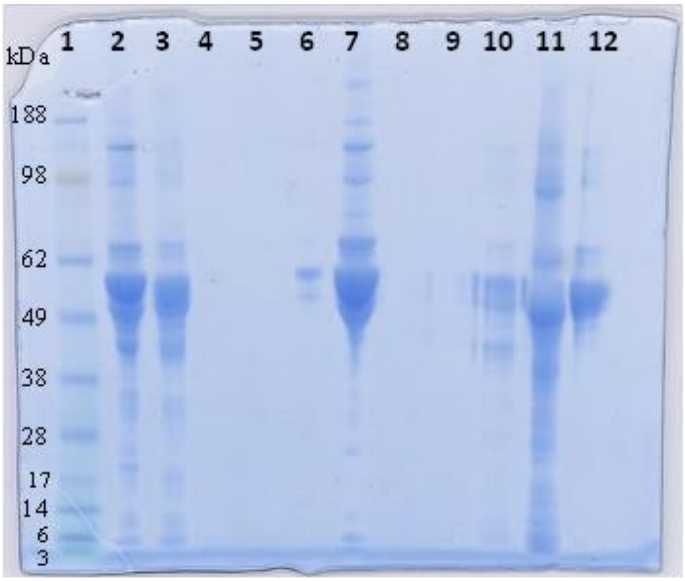


Figure 3 - SDS-PAGE of the pelleted fraction resultant from the supernatant obtained from *C. glabrata* wild type cells grown in SCD plus FBS (lane 2) and of the remainant supernatant from the same culture (lane 3); samples constituted of the supernantant obtained from a *C. glabrata* wild type culture in SCD (lane 4) and of the remainant supernatant from the same culture (lane 5); Control samples with only SCD (lane 6) and with SCD plus FBS (lane 7); Pelleted fraction resultant from the supernatant obtained from *S. cerevisiae* Σ1278b cells grown in SCD (lane 8) and of the remainant supernatant from the same culture (lane 9); samples constituted of the supernantant obtained from a *S. cerevisiae* Σ1278b culture in SCD plus FBS (lane 10) and of the remainant supernatant from the same culture (lane 11); Secretion on *C. glabrata* wild type cells cultured on SCD with agar and FBS (lane 12); Lane 1 - SeeBlue® Plus2 Pre-stained Protein Standard.

3.2 Determination of Culture Secretion Origin

As referred, this project began after the finding of a secretion of unknown composition produced in a *Candida glabrata* culture performed in minimal medium supplemented with glucose and fetal bovine serum (FBS). Facing this, it was important to determine the origin of this secretion and to relate it with the presence of FBS. *C. glabrata* was the species that was chosen for the study.

C. glabrata wild type was cultured in minimal medium containing glucose with or without FBS and it was verified the formation of a dense secretion as a white pellet, only in the presence of FBS (Figure 4).

Figure 4 - The left picture illustrates several colonies of *C. glabrata* cultured in minimal medium with agar and supplemented with glucose and FBS; the right picture represents two falcons with cultures of *C. glabrata* in minimal medium with glucose (A) and *C. glabrata* in minimal medium supplemented with glucose and FBS (B).

To determine if the secretion had proteins on its composition, SDS-PAGE analysis was used because it is a very useful tool to separate protein by molecular size (Brunelle & Green, 2014). After, SDS-PAGE samples were stained with SimplyBlue™ SafeStain (Figure 5). A run containing the samples is shown in Figure 5. The purpose was to see if the proteins present in the secretion were part of the composition of the FBS or if they had been secreted by *Candida glabrata* cells.

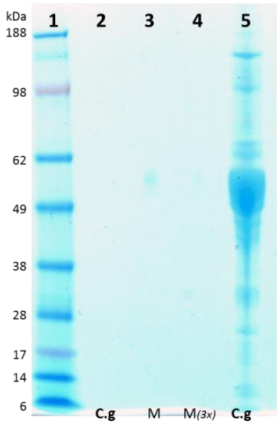


Figure 5 - SDS-PAGE of the pelleted fraction resultant from the supernatants of *C. glabrata* grown in SCD (minimal medium with glucose) (lane 2) and in the presence of FBS (lane 5). Controls were included for SCD + FBS (lane 3) and SCD + FBS (3x diluted) (lane 4). Lane 1 - SeeBlue® Plus2 Pre-stained Protein Standard.

From the results obtained, it is possible to conclude that the pelleted secretion contains some proteins and that those proteins were not part of the composition of the FBS that was pelleted (Figure 5 - lanes 3 and 4) nor the composition of *C. glabrata* cultured on minimal medium (Figure 5 - lane 2). Clearly, as it is possible to observe on the lane 5 (Figure 5), *Candida glabrata* cells cultured on minimal medium plus FBS produce a secretion full of proteins.

In order to concentrate the secreted proteins to allow a better analysis, the supernatant was separated by molecular weight using an Amicon centrifugal filter unit (Ultra-15, MWCO 10 kDa). The different samples (from Amicon subfractions and control without Amicon centrifugation) were analysed by SDS-PAGE and SimplyBlue™ SafeStain (Figure 6).

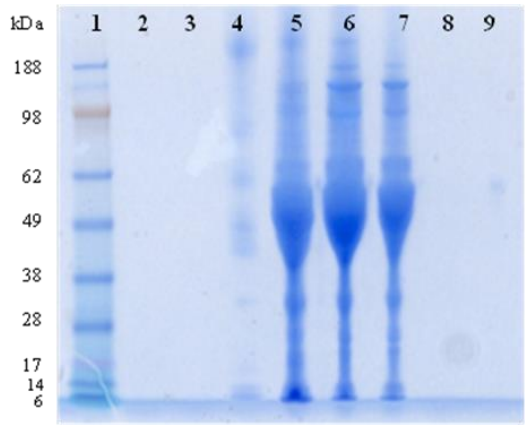


Figure 6 - SDS-PAGE of the pelleted fraction resultant from the supernatants obtained from the Amicon subfraction <10 kDa for cells grown in SCD (lane 2) and with FBS (lane 3); from the Amicon subfraction >10 kDa for cells grown in SCD (lane 4) and with FBS (lane 5); control samples, without Amicon centrifugation, supertantant from cells grown in SCD with FBS (lanes 6 and 7) and without it (lanes 8 and 9). Samples on lanes 7 and 9 are 2x dilutions from samples 6 and 8, respectively. Lane 1 - SeeBlue® Plus2 Pre-stained Protein Standard.

It can be observed that the supernatant from the culture containing FBS presents proteins with a molecular weight higher than 10 kDa (Figure 6 - lane 5) but no proteins with a molecular weight lower than 10 kDa (Figure 6 - lane 3). The supernatant from the culture without FBS (Figure 6 - lanes 2 and 4) contains proteins with a molecular weight higher than 10 kDa (Figure 6 - lane 4) - what was not expected - but no proteins with a molecular weight lower than 10 kDa (Figure 6 - lane 2). This pattern resembles the pattern of the pellets of white secretion of cells grown in presence of FBS (Figure 6 - lanes 6 and 7) and the pattern of the pellets of cells grown without FBS (Figure 6 - lanes 8 and 9), despite the pellet of these last two cultures was not visible.

Because the supernatant contains a similar protein pattern as the secreted pellet, the effect of the pellet dilution into the surrounding medium was tested. The pellet was washed several times with TE-buffer and this was analyzed again by SDS-PAGE and SimplyBlue™ SafeStain (Figure 7). As the washes do not show any proteins, the pattern from the pelleted secretion is truly due to proteins present in this pellet and it is not a remnant of the supernatant.

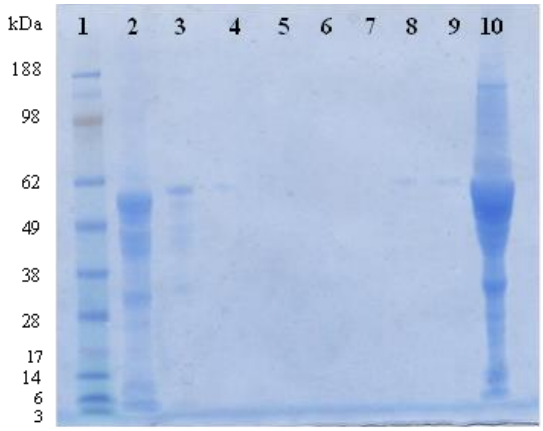


Figure 7 - SDS-PAGE of different washes of the pelleted fraction resultant from the supernatants resulting from *C. glabrata* grown in SCD and FBS; Lane 2 – pelleted supernatant; Lane 3 – first wash of the pelleted supernatant; Lanes 4 to 9 – second to seventh washes of the pelleted supernantant, respectively; Lane 10 – pelleted supertantant after washes. Lane 1 - SeeBlue® Plus2 Pre-stained Protein Standard.

In sum, it was shown that the origin of the secretion was related to the presence of FBS and that there were proteins on the secretion not from the FBS composition but from the *C. glabrata* cells. However, little is known about the secretion itself so a mass spectrometry analysis is required.

3.3 Mass Spectrometry (MS)

Three samples were sent to the SyBioMa facility (KUL) for protein identification using mass spectrometry (LC-MS/MS and Orbitrap Qexactive). This quadrupole orbitrap mass spectrometer possesses a "Q Exactive" instrument that features high ion currents and fast high-energy collision-induced dissociation peptide fragmentation because of parallel filling and detection modes. The image current from the detector is processed by a Fourier Transformation algorithm, doubling mass spectrometric resolution (Michalski et al., 2011). The secretion pelleted from the *Candida glabrata* culture in minimal medium containing serum was analyzed. As a control, similar conditions without FBS were used. A second control consisted of minimal medium containing FBS. All samples were treated identical.

To quantify the peptides in all samples tested, MS technic combined with the software *Progenesis LC-MS* (Nonlinear Dynamics). Data were analysed using *Scaffold*. Comparing the results of the two algorithms used increases the reliability of the protein identification. *Mascot* provides a score for each identified peptide, while using *Scaffold* the fragment spectrum can be investigated and the number of peptides for each gene. After comparing the hits of *Mascot* (scores higher than 40) and *Scaffold* (several peptides per hit and descent a fragmental spectrum) we were able to identify the proteins secreted in presence of serum (Table 4). The targets were blast against all eukaryotic species in the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find homologs. Using the online Signal P 4.1 software (http://www.cbs.dtu.dk/services/SignalP/) it was possible to determine which of the proteins were secreted. This program identifies proteins and evaluates which of them are predicted to have a secretion signal by a Hidden Markov model (Stead et al., 2009).

Table 4 - Significant genes obtained from the mass spectrometry results and secreted proteins by *C. glabrata*

MS hits	***S. cerevisiae* homolog**
CAGL0A02255g	YCL049C
CAGL0G00308g	*SCW4*, *SCW10*
CAGL0J09922g	*SUN4*, *SIM1*
CAGL0M13805g	*SCW4*, *SCW10*
CAGL0K07359g	YMR244W

According to Weig et al. (2004), CAGL0G00308g and CAGL0M13805g are closely related to *C. albicans* Scw1p, as well as to *S. cerevisiae* Scw4 and Scw10p, being a member of the Bg12 family of

cell wall glucanases. In the study of Stead et al. (2009) it was found a predicted secreted glycoprotein: *SUN4*, that is a cell wall protein related to glucanases, possibly involved in cell wall septation.

The higher number of cell wall proteins identified by Weig et al. (2004) comparing with the much lower number of MS hits identified (Table 4), may be because these predicted secreted proteins found may not be expressed under same conditions as in the other published study or are *bona fide* secreted proteins (Stead et al., 2009).

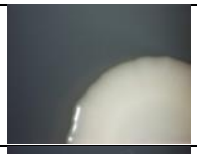
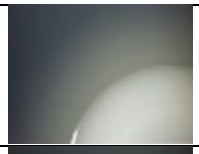
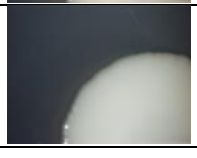
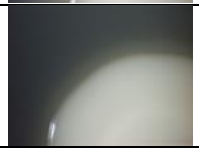
3.4 Screening of the *C. glabrata* Deletion Collection

In order to find the genes responsible for the *C. glabrata* secretion, the deletion collection of *C. glabrata* (Schwarzmüller et al., 2014) was screened for defective secretion. The 619 different strains tested, each lacking one specific gene, represent almost 12% of *C. glabrata* genome (Schwarzmüller et al., 2014).

Several categories such as environmental stress sensing and signaling (MAPK pathways, TOR, RIM, PKA), transcriptional regulation, antifungal drug resistance (PDR network, membrane permeases), cell wall structure and homeostasis (glucan, mannan, chitin synthesis, glycosylation, adhesins, glycosylphosphatidylinositol (GPI)-an- chor), chromatin and histone modification, iron metabolism and metal sensitivity, peroxisome biogenesis and also genes lacking obvious orthologues in *S. cerevisiae*, are present on the collection (Schwarzmüller et al., 2014).

The strains were incubated on minimal medium with agar overnight. Then, these strains were plated on minimal medium supplemented with glucose and FBS and incubated for two days. All strains were able to secrete in presence of FBS (Table 5), indicating that the collection does not contain a gene that is essential for secretion.

Table 5 – Example of two strains (HTL and BG2) from the *C. glabrata* deletion collection used, screened on agar plates with minimal medium and glucose (SCD) and minimal medium and glucose and FBS (SCD + FBS), at 48 hours incubation. It is possible to observe that both strains secreted in the presence of FBS

Strains	SCD	SCD+FBS
HTL		
BG2		

3.5 Determination of the Minimal Inhibitory Concentration (MIC)

This assay was performed to verify if the presence of FBS in the medium changes the MIC value for *Candida glabrata* cells.

Susceptibility tests are most often used when the causative organism is thought to belong to a species capable of exhibiting resistance to commonly used antimicrobial agents, as is the case for *C. glabrata* (Rex et al., 2008). In addition, susceptibility tests may help to optimize antifungal therapy in candidaemia due to non-albicans *Candida* species (Krcmery & Barnes, 2002).

The fluconazole MIC was tested in the presence and absence of FBS for *C. glabrata* wild type cells (ATCC 2001). Following the MIC official guidelines CLSI M27-A3 (Rex et al., 2008), a twofold dilution series from 64 to 0.0625 µg/ml fluconazole was used (Figure 8).

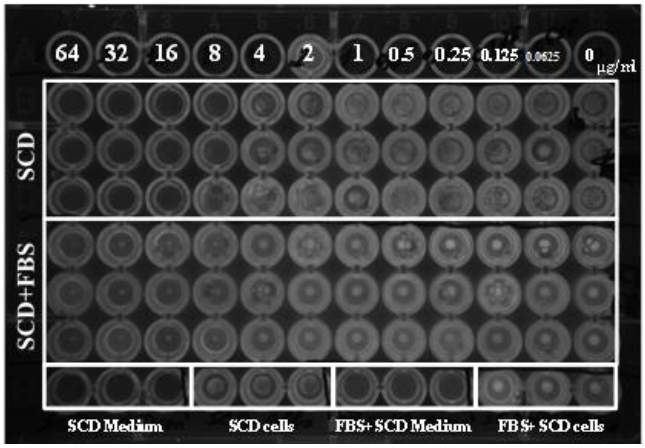


Figure 8 - Image of the 96-well plate containing the MIC assay (24h) to test fluconazole. The first row of the plate is composed of empty wells. In the second to fourth rows there are cultures of *C. glabrata* wild type cells in SCD. In the fifth to seventh rows there are *C. glabrata* wild type cells in SCD plus FBS. The concentration of antifungal in both this groups of rows decreases from well to well till 0 (as according to the information on the first row). The last row of the plate contains the controls (no antifungal), constituted only by minimal medium with glucose (SCD Medium), *C. glabrata* cultured in minimal medium with glucose (SCD cells), minimal medium with glucose supplemented with FBS (FBS+SCD Medium) and *C. glabrata* cultured in minimal medium with glucose supplemented with FBS (FBS+SCD cells), respectively.

Cells cultured in minimal medium without serum had a MIC of 8 µg/ml, which is in accordance with the results of Kucharíková et al. (2014) on the effect of fluconazole on the growth of planktonic cells of *Candida glabrata* (ATCC 2001).

Interestingly, it was not possible to determine the MIC for the cells cultured in minimal medium containing serum. The effect of serum on polyene cytotoxicity had already been reported by (Kinsky, 1963). It seemed at the time that the inhibitory effect of serum might have been a consequence of polyene binding by a component in the serum. That binding probably resulted in a reduction of the effective antimicrobial concentration and would explain why amphotericin B was beneficial in the treatment of systemic fungal infections without serious effects (Kinsky, 1963). A following study by Bonner et al. (1976) concluded that FBS was found to antagonize the *in vitro* antifungal effects of amphotericin B, as higher levels of antibiotic were required to inhibit the growth of *S. cerevisiae* by 50%. As fluconazole leads to a diminished ergosterol production (Rodrigues et al., 2013) and since *C. glabrata* is closely related to *S. cerevisiae* (Roetzer et al., 2011), one possible hypothesis is that, as for amphotericin B, also fluconazole cytotoxicity is maybe affected by the presence of FBS, leading to higher MIC values for *C. glabrata* cultured in minimal medium supplemented with FBS.

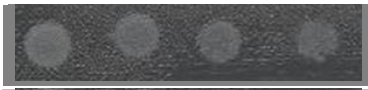
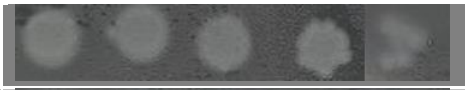
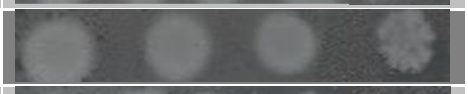
In addition, there was a colony morphology difference for the two conditions tested (Figure 8). In absence of FBS, colonies of the cells were dispersed over the plastic surface and in presence of FBS, the cells formed one central colony. So it can be concluded that serum triggers cell-cell adhesion in *C. glabrata*, although the mechanism behind is not elucidated yet.

3.6 Susceptibility Testing with Congo Red and Calcofluor White

The susceptibility of *Candida glabrata* (ATCC 2001) cells to calcofluor white (CW) and Congo red (CR) was evaluated in the presence and absence of FBS. These two anionic dyes interfere with the construction and stress response of the fungal cell wall, causing stress (Ram & Klis, 2006).

Plates with minimal medium (2% glucose) in presence or absence of 5% FBS were supplemented with CW (100 and 200 µg/ml) or CR (1 and 3 mg/ml). Then, a tenfold dilution series of *C. glabrata* ATCC 2001 was spotted and incubated at 37 °C (results are shown in Table 6).

Table 6 - Result of spot assay (24 and 48h) in minimal medium with glucose (SCD) (A) or SCD supplemented with 5% FBS (SCD + FBS) (B) for CW and CR

Conditions			24 hours	48 hours
Control		(A)		
		(B)		
CW	100 µg/ml	(A)		
		(B)		
	200 µg/ml	(A)		
		(B)		
CR	1 mg/ml	(A)		
		(B)		
	3 mg/ml	(A)		
		(B)		

In general, the colonies in presence of FBS are bigger, compared to the condition in the absence of FBS. This is in line with the observation that the cell suspension spots in presence of FBS are diffusing more over the agar plate.

Regarding CW, there is a slightly growth difference between the controls and the two different concentrations used: 100 µg/ml and 200 µg/ml. The inhibition of growth seems to be bigger as the

CW concentration used increases, both in presence and absence of serum. Therefore, the presence of FBS has no significant effect on the response to CW.

Regarding CR, there is again a growth difference between the controls and the two different concentrations used: 1 and 3 mg/ml. There is higher inhibition of growth as the concentration of CR used increases, what was not expected according to the paper of Vandeputte et al. (2007). These authors were not able to achieve inhibition even at the highest CR concentration used of 4 mg/ml. In the presence of FBS on culture, it seems to be a little inhibition on the growth but not distinguishable between the two different concentrations used.

From the resulting CW and CR assays, it can be concluded that the presence of FBS has no remarkable effect on the two cell wall stress factors tested. The reason for that is not elucidated yet.

3.7 *Candida glabrata* Adhesion

Cell adhesion is the ability of a single cell to stick to another cell or an extracellular matrix (ECM) (Khalili & Ahmad, 2015) or even a material. *Candida glabrata* adhesion is important for its virulence, since it mediates the attachment of the cells to the host as the first step of infection and secondly the attachment cell to cell.

The adhesion assay was performed to investigate if *C. glabrata* cells were affected by the presence of different concentrations of FBS (0, 1, 2, 5, 10 and 20%) (Figure 9).

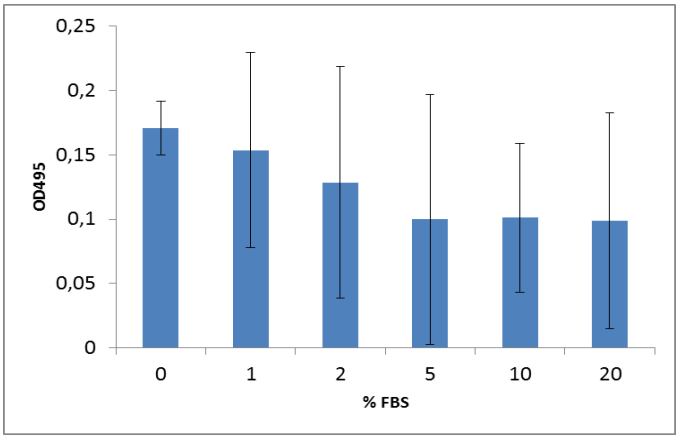


Figure 9 - *In vitro* adhesion of *C. glabrata* ATCC 2001 analyzed by growing cells supplemented with different percentages of FBS in 96-wells plate. Nonadherent cells were washed away with PBS, and remaining adherent cells were stained with XTT menadione solution.

In this assay (Figure 9), as the percentage of FBS in the *C. glabrata* culture increase, the adherence of *C. glabrata* cells tends to decrease (despite the standard deviation is too high to be possible to draw a faithful conclusion about this result).

According to the results of Ding et al. 2014, that concluded that non-protein components in the human serum inhibited the adhesion of *C. albicans* cells, it is possible to hypothesis that like human serum, also FBS inhibits the *C. glabrata* cells adhesion.

The XTT method was chosen being the most accurate and reproducible assay from the listed quantification protocols as showed in Table 7.

Table 7 - Summarization of the various factors influencing each quantification protocol; (-) disadvantage, (+) advantage (Taff et al., 2012)

	Accuracy	**Reproducibility**	**Time**	**Cost**
XTT	+ + +	+ + + +	+	+ +
Crystal Violet	+	+ + + +	+ +	+
CFU	+ +	+ + + +	+ + +	+ +
DNA	-	+ + +	+ +	+ + +
Amplification	-	+ +	+ + +	+ + + +
BCA Protein	+	+	+	+
Dry Cell Weight	-	+ +	+ +	+

Because *Candida* species can form biofilms on intravenous catheters and other inserted medical devices that may come into contact with blood, serum is regarded as an external cue to trigger cell adhesion and biofilm formation (Ding et al., 2014). For that reason the adhesion of *C. glabrata* cells was tested on the presence of FBS.

4. CHAPTER IV

Conclusion and Future Work

Nowadays, *Candida* species are the fourth most common cause of nosocomial bloodstream infections (BSI) in USA. Despite that, *Candida albicans*, the most common pathogen causing this type of infections, has been becoming less dangerous than before. On the basis of that is the exhaustive study of this species that led to effective antimicrobial therapies and to an increasing rate of success on the treatment of people infected. It is completely fair to state that, nowadays, the number *of C. albicans* infections has been stabilizing.

However, *Candida glabrata*, that like *C. albicans,* is a yeast that takes part of the human's microbial flora as an opportunistic pathogen, has been emerging to become an important and dangerous nosocomial pathogen. In contrast to *C. albicans*, the number of *C. glabrata* infections has been increasing significantly on the last three decades, making this pathogen of increasing importance. One big problem of this "new" *Candida* species is that has different virulence factors than *C. albicans*. So, before the scientific community knew about this new *Candida*, they would just treat it like it was a "regular" *Candida albicans*, and that led to increasing resistance and mortality rates. The reason is just because *C. glabrata* is resistant to azoles, the most common class of antimicrobials used to treat *C. albicans* infections. This way, the delay on the diagnosis and on the administration of the right drug made impossible for some infected persons to survive.

On the other hand, one good advance was the discovery that *C. glabrata* is closely related to *Saccharomyces cerevisiae*. One way that could allow a better understanding of this yeast virulence mechanism was to compare it with *S. cerevisiae*, not only on them resemblances but on their differences too. For instance, analysing differences in both species genomes, it is possible to find genes important for virulence like the *EPA* genes that are not present in *S. cerevisiae* genome and have an important role in *C. glabrata* virulence.

Taking all this points together, it is of major importance that an efficient antifungal drug against this pathogen is design.

Like already exposed, the main evidence for this project was the observation that there was a secretion of unknown composition identified on *C. glabrata* cultures in minimal medium with glucose and FBS but not in the absence of FBS. Facing this, the goals were to evaluate if the secretion occurred for other species and to evaluate the role of FBS in this secretion production and to characterize the composition of that secretion.

This way, several strains of *C. glabrata*, *S. cerevisiae* and *C. albicans* were spotted on minimal medium with glucose and FBS. The results showed that all the strains from all species secreted only in the presence of FBS, what was not expected.

As *C. glabrata* is closely related to *S. cerevisiae*, a posterior SDS-PAGE was performed to compare in more detail the secretions produced by this species. The results were inconclusive because there were more proteins on the remainant supernatant of *S. cerevisiae* strain than in the pelleted supernatant, in opposition to *C. glabrata* strain, what makes it difficult to compare both. However, it seems that *S. cerevisiae* strains have more susceptibility to oxidative stress than the *C. glabrata* strains, what make them secrete more than the last.

Then it was decided to study the secretion of *C. glabrata* strains. The SDS-PAGE results confirmed that the secretion only occurred with the presence of FBS in the medium where the cells were cultured. In addition, it showed that the proteins on the secretion were not part of the proteic composition of the FBS but came from the cells. The way the cells were affected by the presence of the FBS is still unclear. Recurring to a similar assay complemented with an Amicon centrifugal unit, it was shown that the molecular weight of the proteins was bigger than 10 kDa.

Then, as it was already clear which was the origin of the secretion, it was important to determine the composition of that secretion. The mass spectrometry results were five genes that had homology on *S. cerevisiae* cell wall glucanases. Recurring to Signal P 4.1 software it was possible to confirm that these proteins had been secreted.

As the origin of the secretion and the composition of that secretion were already investigated, a deletion collection was screened next to try to determine the gene(s) responsible for the secretion of *C. glabrata* cells on the presence of FBS. Unfortunately, all genes produced secretion, so it was not possible to determine that/those gene(s).

With the purpose of evaluating the effect of FBS on the MIC of *C. glabrata* cells, a MIC assay in the presence of fluconazole was performed. It was possible to verify a morphological difference between the cells grown in FBS and without it. So, serum triggers cell-cell adhesion in *C. glabrata*, although the mechanism is not elucidated yet. In addition, the MIC value increased in the presence of FBS. This makes it possible to hypothesis that fluconazole cytotoxicity is affected in the presence of FBS, leading to higher MIC values. The way the FBS affects the MIC is not elucidated. Another susceptibility testing was performed then with CR and CW, however, the conclusion was that the FBS did not have any remarkable effect on the two cell wall stress dyes tested. The explanation behind that result is still unclear.

Adhesion assay was performed, and the results showed that with the increasing concentration of FBS, the cell adhesion would decrease. In hypothesis, this seems to be according to the decrease in

C. albicans adhesion with the presence of human serum. However, more repetitions of this assay were needed as the standard deviation was too high to make faithful conclusions.

In the future, maybe it would be important to screen a *S. cerevisiae* deletion collection. As there are more genes already found and studied, and as this species is closely related to *C. glabrata*, maybe it would be possible to find the gene responsible for the secretion. In addition, it was important to evaluate the behaviour of the cells with the increasing concentrations of CR and CW, as the concentrations used didn't have a remarkable effect on the cultures supplemented with FBS. Also, bigger concentrations of fluconazole should be tested, to try to find the MIC value for *C. glabrata* cells cultured in the presence of FBS. Other classes of antifungals should be tested too like the polyene amphotericin B and the echinocandins: micafungin, caspofungin and anidulafungin. In addition, the expression of the genes found should be tested.

Despite the secretion in the presence of FBS is not exclusive for *C. glabrata* cultures, it stills something that should be more investigated. It seems the cell wall proteins of *C. glabrata* are affected, so, maybe it can be of major importance to exhaustibly study the target of the FBS that makes it possible for the cells to secrete. In a hypothetical future, the gene responsible for the secretion on *C. glabrata* cells can be the perfect target for the most effective antifungal drug against this pathogen, what could help to save lives.

5. CHAPTER V

References

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