Universidade do Minho
Escola de Engenharia

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Clinical *Candida* species co-infection and associated virulence

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Trabalho efetuado sob a orientação da
Doutora Mariana Contente Rangel Henriques
e do
Doutor David Wynne Williams

março de 2014

"Caminhante, o caminho são os teus passos e nada mais.

Caminhante, não há caminho. O caminho faz-se caminhando.

E ao olhar para trás, vês os passos que nunca voltarás a pisar."

In Campos de Castilla

Antonio Machado, 1910

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Chegou o momento de agradecer a todas as pessoas que fizeram com que o chegar à meta de mais esta minha caminhada não fosse apenas um sonho. **Hoje esse sonho tornou-se realidade**.

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Abstract/Sumário

Abstract

The *Candida* genus consists of approximately 200 species of fungi and collectively represents a highly heterogenic group. Clinically, the most important specie is *Candida albicans,* an opportunistic fungal pathogen of humans that frequently causes superficial infections of oral and vaginal mucosal surfaces of debilitated individuals. This microorganism is, however, also commonly encountered as a commensal in healthy individuals where it is a component of the normal microflora. Nowadays, non-*Candida albicans Candida* (NCAC) species such as *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* are also becoming frequently identified as potential human pathogens. Thus, the principal aim of this thesis was to obtain significant insight into the virulence mechanisms of *Candida* species, with special relevance to those colonising the vaginal tissue, as well as to evaluate their resistance to new antifungal agents.

The treatment of human infections caused by *Candida* (candidosis) is difficult, especially due to the eukaryotic nature of fungal cells. Furthermore, several *Candida* species exhibit both intrinsic and acquired resistance to common antifungal agents and biofilms produced by *Candida* are also less susceptible. Thus, the first goal of this thesis was to perform a screening of the antifungal potential of natural plant extracts (*Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* and *Cistus ladanifer*) and four phenolic compounds (gallic acid, catechin, luteolin and quercetin), identified from these plants, against *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. The minimum inhibitory concentration (MIC) of plant extracts and phenolic compounds was determined according to standard methods. The antifungal potential of the phenolic compounds was also tested against *Candida* biofilms, and was assessed by quantification of colony forming units (CFUs). Overall, all plant extracts, as well as the phenolic compounds, especially gallic acid, revealed promising antifungal activity against *Candida* species. However, only gallic acid and quercetin demonstrated a slight effect against *Candida* species biofilms.

Although the majority of the studies regarding *Candida* infection of human mucosal surfaces often include only single species, often candidosis is associated with mixed colonisation. The second aim of this research was to examine the interactions and

expression of virulence factors by *C. albicans* and *C. glabrata* using a reconstituted human vaginal epithelium (RHVE). The pathogenesis of *C. albicans* and *C. glabrata* single and co-infections were investigated using peptide nucleic probe fluorescent *in situ* hybridization (PNA FISH), confocal laser scanning microscopy (CLSM) and a novel qRT-PCR protocol for *Candida* quantification in the tissues. RHVE damage was evaluated by measuring lactate dehydrogenase activity. *Candida* virulence gene (*HWP1*, *ALS*, *EPA*, *PLB*, *PLD* and *SAP*) expression was evaluated by qRT-PCR. It was shown that although *C. albicans* was a higher coloniser and invader of vaginal tissue, than *C. glabrata,* the invasiveness of *C. glabrata* strains was enhanced in the presence of *C. albicans*. Additionally, the results suggest an important role of *HWP1*, *PLD1* and *ALS3* virulence factors in *C. albicans* and *C. glabrata* pathogenicity.

It is known that several environmental factors may be altered *in vivo*, to facilitate the conversion of *Candida* from a harmless commensal to a pathogenic organism. In the vaginal environment, hormonal changes are amongst such influencing factors. Therefore, the effect of progesterone (hormone) in *C. albicans* biofilm formation and RHVE colonisation and invasion was examined, using the same techniques described above. It was found that progesterone decreased the capacity of *C. albicans* to form biofilms and it was shown that *C. albicans* was a higher RHVE coloniser in the absence of progesterone. Gene expression by *C. albicans* infecting the vaginal epithelium suggests an important role of *BCR1* and *HWP1* virulence factor in *C. albicans* pathogenicity.

In summary, this work emphasised the importance of studying new natural products as potential antifungal agents and also provided more insight into the mechanisms of vaginal infections caused by *Candida* species, in mono and mixed cultures, as well as in the presence of progesterone.

Sumário

O género *Candida* consiste em aproximadamente 200 espécies de fungos que em conjunto representam um grupo heterogéneo. A espécie clínica mais importante é *Candida albicans*, um oportunista patogénico para os humanos, que causa frequentemente infeções superficiais da mucosa oral e/ou vaginal em indivíduos débeis. Este microrganismo é, contudo, também comummente encontrado como comensal em indivíduos saudáveis onde é um componente da sua microflora. Contudo, hoje em dia espécies de *Candida* não *Candida albicans* (NCAC), como a *Candida glabrata,* a *Candida parapsilosis* e a *Candida tropicalis* começam a ser identificadas como patogénicas. Assim, o principal objetivo desta tese foi obter novos conhecimentos sobre o mecanismo de virulência das espécies de *Candida*, com especial relevo para a sua colonização do trato vaginal, assim como determinar a resistência a novos agentes antifúngicos.

O tratamento de infeções humanas causadas por espécies de *Candida* (candidíases) é difícil, especialmente devido à natureza eucariota das células fúngicas. Para além disso, muitas espécies de *Candida* exibem resistência intrínseca e/ou adquirida aos antifúngicos comuns e os seus biofilmes são também menos suscetíveis. Assim, o primeiro objetivo desta tese foi realizar uma avaliação do potencial antifúngico de extratos naturais de plantas (*Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* e *Cistus ladanifer*) e de 4 compostos fenólicos (ácido gálico, catequina, luteolina e quercetina) identificados nessas plantas contra *C. albicans*, *C. glabrata*, *C. parapsilosis* e *C. tropicalis*. A concentração mínima inibitória (MIC) dos extratos das plantas e dos compostos fenólicos foi determinada seguindo as normas. Para além disso, o potencial antifúngico dos compostos fenólicos foi avaliado em biofilmes de *Candida*, pela quantificação do número de unidades formadoras de colónias. Globalmente, todos os extratos de plantas e os compostos fenólicos, especialmente o ácido gálico, revelaram potencial antifúngico contra as espécies de *Candida*. Contudo, só o ácido gálico e a quercetina demonstraram um pequeno efeito contra os biofilmes de *Candida.*

Embora as infeções da superfície das mucosas sejam normalmente estudadas com apenas um microrganismo, sabe-se que em muitas situações as candidíases

ocorrem devido a uma infeção mista de *Candida*. Assim, o segundo objetivo foi examinar as interações e a expressão de fatores de virulência de *C. albicans* e *C. glabrata* em infeções simples e mistas, utilizando um epitélio vaginal humano reconstituído (RHVE). Para tal, usaram-se sondas de ácidos nucleicos de hibridação *in situ*, microscopia de confocal laser e a técnica de PCR em tempo real para a quantificação do número de células de *Candida* presentes no tecido. O dano do RHVE foi avaliado pela atividade da enzima lactato desidrogenase. A expressão genética (*HWP1*, *ALS*, *EPA*, *PLB*, *PLD* e *SAP)* da virulência da *Candida* foi avaliada por PCR quantitativo. Então, foi possível verificar que apesar de *C. albicans* ser muito mais colonizadora e invasora do tecido vaginal, do que a *C. glabrata,* a invasão das estirpes de *C. glabrata* aumentou na presença da *C. albicans.* Para além disso, os resultados obtidos sugerem um importante papel dos genes *HWP1*, *PLD1* e *ALS3* na patogenicidade da *C. albicans* e da *C. glabrata.*

Sabe-se que muitos fatores ambientais podem alterar a facilidade de conversão da *Candida* de comensal para patogénica. No ambiente vaginal, as hormonas são conhecidas por serem um desses fatores. Por isso, foi avaliado o efeito da progesterona na formação de biofilmes e colonização do RHVE por *C. albicans*, utilizando as técnicas descritas anteriormente. Assim, mostrou-se que a presença de progesterona diminui a capacidade da *C. albicans* formar biofilme e colonizar o RHVE. Os resultados da expressão genética sugerem um importante papel dos genes *BCR1* e do *HWP1* na patogenicidade da *C. albicans.*

Em suma, este trabalho realçou a importância do estudo de novos produtos naturais como potenciais agentes antifúngicos e permitiu ainda alcançar novos conhecimentos no mecanismo das infeções vaginais causadas por *Candida,* em culturas simples e mistas, bem como na presença de uma hormona.

Publications within the thesis

Publications within the thesis

Papers in peer reviewed journals:

Published:

Carlos Tiago Alves, Isabel C.F.R. Ferreira, Lillian Barros, Sónia Silva, Joana Azeredo, Mariana Henriques. 2014. Antifungal activity of phenolic compounds identified in flowers from the North Eastern Portugal against *Candida* species. **Future Microbiology** 9(2), 139-146.

Lillian Barros, Carlos Tiago Alves, Montserrat Dueñas, Sónia Silva, Rosário Olieveira, Ana Maria Carvalho, Mariana Henriques, Celestino Santos.Buelga, Isabel C.F.R. Ferreira. 2013. Characterization of phenolic compounds in wild medicinal flowers from Portugal by HPLC-DAD-ESI/MS and evaluation of antifungal properties. **Industrial Crops and Products** 44, 104-110.

Lillian Barros, Montserrat Dueñas, Carlos Tiago Alves, Sónia Silva, Mariana Henriques, Celestino Santos.Buelga, Isabel C.F.R. Ferreira. 2013. Antifungal activity and detailed chemical characterization of Cistus ladanifer phenolic extracts. **Industrial Crops and Products** 41, 41– 45.

Submitted:

Carlos Tiago Alves, Sónia Silva, Leonel Pereira, Joana Azeredo, David W. Williams, Mariana Henriques. 2014. Effect of progesterone on *Candida albicans* vaginal pathogenicity.

Carlos Tiago Alves, Xiao-Qing Wei, Sónia Silva, Joana Azeredo, Mariana Henriques, David W. Williams. 2014. *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium.

Other scientific output

Posters in conferences:

Carlos Tiago Alves, Isabel C.F.R. Ferreira, Lillian Barros, Sónia Silva, Rosário Oliveira, Mariana Henriques. 2012. Antifungal activity of natural extracts from North Eastern Portuguese flowers against *Candida* biofilms. **Biofilms 5 - International Conference**, Paris, France, 10th-12th December, (228), 157-158.

Carlos Tiago Alves, Isabel C.F.R. Ferreira, Lillian Barros, Sónia Silva, Rosário Oliveira, Mariana Henriques. 2012. Natural extracts from wild flowers used in Portuguese folk medicine like a new antifungal agents against *Candida* species. **11th ASM Conference on Candida and Candidiasis**, San Francisco, USA, 29th March - 2nd April, (7A), 75-76.

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Nomenclature

Symbols

°C- Degrees

g- Gravity

%- Percent

P- Significance value

Ct- Threshold cycle

$- United State of America Dollar

Abbreviations

Abs- Absorbance

***ACT1*_alb**- Actin *Candida albicans*

***ACT1*_gla**- Actin *Candida glabrata*

AIDS- Acquire Immune Deficiency Syndrome

ALS- Agglutinin like sequence gene

ANOVA- Analysis of variance

AP- Alternative pathway

ATCC- American Type Culture Collection

BCR- Biofilm and Cell Wall Regulator

cDNA- complementary Deoxyribonucleic Acid

CFU- Colony Forming Units

CHROMagar- Chromogenic Media agar

CLSM- Confocal Laser Scanning Microscopy

CV- Crystal Violet

DAPI- 4',6-diamidino-2-phenylindole

DNA- Deoxyribonucleic Acid

dNTP- deoxynucleoside triphosphate

ECM- Extracellular matrix

EPA- Epithelial adhesin gene

Epa- Epithelial adhesin gene protein

EPS- Extracellular polymeric substances

FCT- Fundação para a Ciência e Tecnologia

FFPE- Formalin-Fixed Paraffin-Embedded

FH- Factor H

FISH– Fluorescent *in situ* Hybridization

GPI- Glycophosphatidylinositol anchor protein

h- hour

HIV- Human Immunodeficiency Virus

HLP- (*Haemolysin Like Protein*)

HPLC- High Performance Liquid Chromatography

HWP- Hyphal Wall Protein

kDa- Dalton

LDH- Lactate Dehydrogenase

LIP- Lipases

Log- Logarithm to base 10

MIC- Minimal Inhibitory Concentrations

min- minute

mRNA- messenger Ribonucleic Acid

NaCl- Sodium chloride

NCAC- Non-*Candida albicans Candida*

NCBI- National Center for Biotechnology Information

ND- No Detected

PBS- Phosphate Buffer Saline

PCR- Polymerase Chain Reaction

PLs- Phospholipases

PNA–Peptide Nucleic Acid

qRT-PCR- quantitative Real Time-Polymerase Chain Reaction

RHOE- Reconstituted Human Oral Epithelium

RHVE- Reconstituted Human Vaginal Epithelium

RNA- Ribonucleic Acid

Rev/min- Revolutions per minute

RPMI- Roswell Park Memorial Institute media

rRNA- ribosomal Ribonucleic Acid

SAP- Secreted aspartly proteinase *Candida albicans* gene

Sap- Secreted aspartly proteinase protein

SAPP- Secreted aspartly proteinase *Candida parapsilosis* gene

SAPT- Secreted aspartly proteinase *Candida tropicalis* gene

SDA- Sabouraud Dextrose Agar

SDB- Sabouraud Dextrose Broth

SEM- Scanning Electron Microscopy

v- volume

VC- Vaginal Candidosis

w- weight

YNB- Yeast Nitrogen Base

Aim and Outline of the thesis

Aim of the thesis

The present research aims to provide significant insight into mechanisms of virulence, the importance of *Candida* species co-infection of vaginal epithelium and antimicrobial susceptibility of *Candida* species.

Considering the relatively limited number of available antifungal agents and the increasingly apparent resistance of *Candida* to those that are available, it is essential to identify new and effective antifungal compounds.

The first aim of this study was therefore to identify the antifungal potential of natural plants extracts, of flowers originating from the North Eastern of Portugal and also associated phenolic compounds. Antifungal screening was undertaken against planktonic and biofilms cells of *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*.

Vaginal candidosis (VC) is a fungal infection mainly caused by the opportunistic pathogen, *C. albicans.* However, it is known that VC can involve more than one species, but interaction between these is poorly described. Consequently, the second goal of this research was to examine the interactions and expression of virulence factors by *C. albicans* and *C. glabrata* during mixed species infection. To achieve this, a reconstituted human vaginal epithelium (RHVE), based upon the A431 vaginal squamous cell carcinoma cell line was used to assess mechanisms of tissue degradation and virulence gene expression following infection with *Candida* species.

Several *in vivo* environmental factors (*e.g.* hormone levels, pH, nutrient availability) may alter and facilitate transition of *Candida* from a harmless commensal to a pathogenic organism. Additionally, *Candida* species can rapidly respond to environmental changes and this flexibility may allow these organisms to take advantage of impaired immunity in debilitated patients and therefore facilitate establishment of disease. Therefore, as a final goal, the effect of progesterone on *C. albicans* biofilm formation and RHVE infection was evaluated.

Outline of the thesis

The presented thesis reports research undertaken at CEB - Centre of Biological Engineering, University of Minho, Braga, Portugal, and at the Department of Tissue Engineering & Reparative Dentistry, School of Dentistry, Cardiff University, Cardiff CF4 4XY, United Kingdom, under the supervision of Professor Mariana Henriques and Professor David W. Williams, respectively.

This thesis is organised into five chapters:

The **first chapter** is a general introduction that encompasses the current knowledge of *Candida albicans* and Non-*Candida albicans Candida* species in relation to taxonomy, epidemiology, virulence factors, antifungal resistance and methods of strain differentiation. A description of reconstituted human epithelium models used for demonstrating initial interactions of *Candida* species with human cells is also provided.

In **chapter two,** research involving assessment of the antifungal potential of specific natural plant extracts and phenolic compounds identified in wild plants from North Eastern Portugal as new strategy against *Candida* resistant cells, is described.

The **third chapter** addresses the determination of single and co-species colonisation and invasion of a reconstituted human vaginal epithelium (RHVE) model using *C. albicans* and *C. glabrata* strains. Expression of genes associated with Candida virulence, namely *HWP1*, *ALS*, *EPA*, *PLB*, and *PLD* are also investigated.

The **fourth chapter** presents the effect of progesterone on *C. albicans* biofilm formation and RHVE infection, as well as, its influence in virulence gene expression.

The **fifth** and last **chapter** covers the major conclusions of the thesis, suggesting some important issues that should be explored in future work.

Chapter I

General introduction

1.1. *Candida* species

1.1.1. *Candida albicans* and non-*Candida albicans Candida* species

Of the fungi regarded as human pathogens, members of the genus *Candida* are the most frequently recovered from fungal infections and these *Candida* infections are collectively referred to as candidosis. The genus *Candida* is composed by an extremely heterogeneous group of over 150 species [1], but it is well established that only a few of these are implicated in human candidosis. Additionally, it is known that approximately 65% of the *Candida* species are unable to grow at a temperature of 37°C, and growth at this temperature is an important factor for an organism to be have pathogenic properties [1]. Of the *Candida* species isolated from humans, *Candida albicans* is the most prevalent (80% of isolates) in healthy and infected individuals [2–4], and this species is an endogenous commensal of the gastrointestinal and urogenital tracts [5].

The prevalence of opportunistic fungal infections (candidosis) has dramatically increased over the recent decades and this is particularly evident in immunocompromised individuals [4]. Additionally, in recent decades, coupled with the improvements in the diagnostic methods and the emergence of molecular techniques, new *Candida* species (*i.e.* Non-*Candida albicans Candida* (NCAC) species) have been reported in human fungal infections, particularly *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis, Candida krusei, C. lusitaniae* and *C. dubliniensis* [6–8].

All species produce blastoconidia, which may be round or elongated yeast cells [9]. Most members of the genus also produce a filamentous type of growth, and these are typically pseudohyphae in NCAC, although *C. albicans* and *C. dubliniensis* can produce true hyphae. The difference between hyphae and pseudohyphae is related to the way they are formed (Figure 1.1). Pseudohyphae are formed from yeast cells or from hyphae by budding. This new structure remains attached to the parent cell and elongates, forming filaments with constrictions and without septa (internal cross walls). In the case of true hyphae, these filaments are formed from yeast cells or from hyphae branches. The development of true hyphae begins as outgrowths (germ tubes) from the yeast cell,

followed by elongation and branching of the filament. Septa are also created to provide structural support and compartmentalised sections within the hyphae of fungi [10,11].

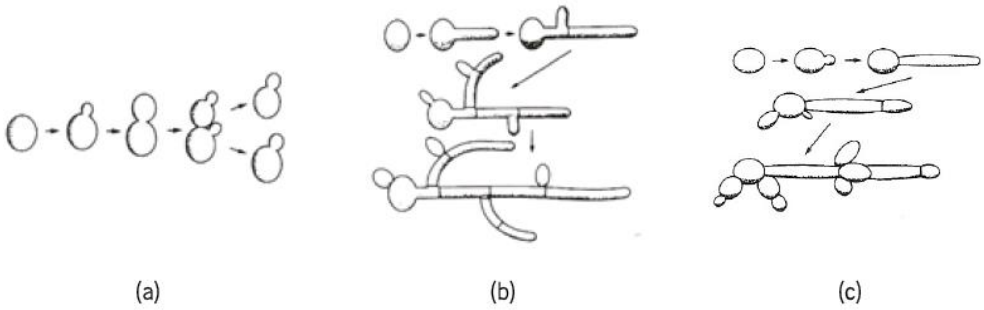

Figure 1.1 - The growth forms of *Candida* species: growth as yeasts **(a)**, production of true hyphae **(b)** and pseudohyphae **(c)**. Adapted from [11].

C. albicans and *C. dubliniensis* are considered polymorphic, due to their ability to form yeast, hyphae and pseudohyphae, and are also referred to as being germ tube positive [1]. However, *C. glabrata* is not polymorphic and grows only as blastoconidia (yeast). By this criterion, this species was only classified in the genus *Candida* in 1978, as it was only at this time that the ability to form pseudohyphae was recognised as not being a reliable distinguishing factor for members of genus *Candida* [12]. Compared with *C. albicans, C. parapsilosis* does not produce true hyphae, producing only pseudohyphae [9]. *C. tropicalis* can also produce oval blastospores, hyphae and pseudohyphae [1,9]. Yeast size should also be highlighted, as *C. glabrata* yeast (1-4 μm) are considerably smaller than *C. albicans* (4-6 μm), *C. tropicalis* (4-8 μm), and *C. parapsilosis* (2.5-4 μm) [11].

In terms of biochemical characteristics of these species, it is important to note that *C. albicans* has the ability to ferment or assimilate a large group of sugars with the exception of sucrose, while the NCAC species ferment and assimilate specific sugars: glucose and trehalose, in the case of *C. glabrata*, and sucrose and maltose, for *C. tropicalis*. It is also described that *C. parapsilosis* does not have the ability to ferment maltose [13].

Through genetic characterisation, differences between some NCAC species and *C. albicans* are clearly evident. Indeed, *C. glabrata* and *C. lusitaniae* are actually haploid

species, while *C. albicans* and several NCAC species *e.g. C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* are diploid. It is also reported that *C. tropicalis* is more similar to *C. albicans* than *C. glabrata* [14].

The different *Candida* species can also be easily distinguished by colony color on chromogenic agar medium (*e.g.* CHROMagar® Candida) [10]. Macroscopically, colonies of *Candida* species are cream colored to yellowish in Sabouraud's Dextrose Agar (SDA). On CHROMagar® *Candida* (Figure 1.2), *C. albicans* produces light green colonies, *C. tropicalis* dark-blue colonies, *C. glabrata* white or pink-purple colonies, whereas *C. parapsilosis* produces cream coloured colonies [15].

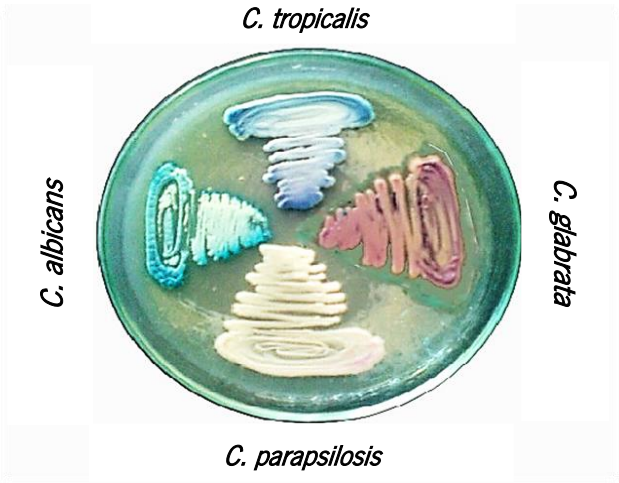

Figure 1.2 - Colonies of *Candida* species grown for 48 h on CHROMagar®*Candida* at 37°C. Adapted from [16].

The cell wall of *Candida* species is an essential component of its success as a pathogen [17]. It is a dynamic and highly organized structure that determines both cell shape and viability, as well as providing a permeability barrier and determining the interaction between the microorganism and its environment [18]. The cell wall is required for growth, provides strength and protection against osmotic insult and might be considered an important factor in host tissue invasion and colonisation. The *Candida* cell wall is also the first point of contact with immune system of the host and therefore plays an important role in recognition and phagocytosis by host immune cells [18].

The *C. albicans* cell wall is mostly composed by polysaccharides (80%); with β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin giving a robust external structure and scaffold for the glycoprotein layer (20%) (Figure 1.3). The glycoprotein layer comprised of remaining cell wall constituents including lipids, various inorganic salts and also many proteins (3.3-3.7%) and mannose (24.3-28.9%). These components are involved in permeability control, interaction with host cells, recognition of other fungi and regulation of several processes [19]. Than compared with *C. albicans*, *C. glabrata* cell wall has a higher quantity of proteins and mannose and a lower quantities of chitin and glucans [20].

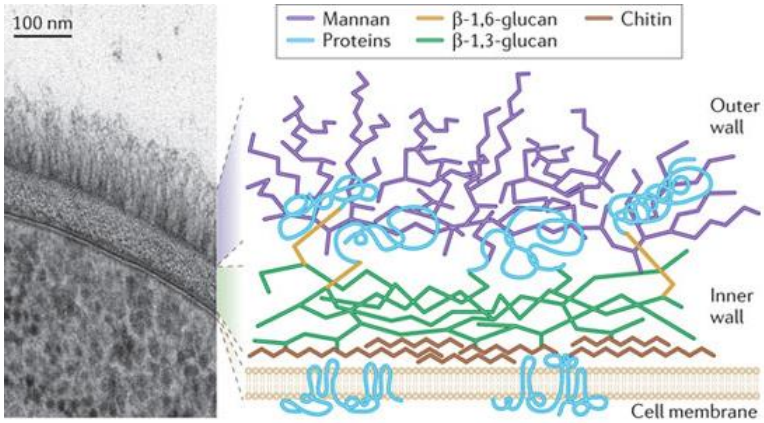

Figure 1.3 - Section of the *C. albicans* cell wall imaged by transmission electron microscopy and a schematic depiction of the arrangement of the major components of cell wall [21].

1.1.2. Health Associated Infections and Co-infections

Candida species normally exist as commensals, but can also be opportunistic pathogens. *Candida* primarily causes superficial infections, affecting the oral and vaginal mucosa, but they can also be responsible for several systemic infections. The prevalence of opportunistic fungal infections has increased dramatically over recent decades and this is particularly evident in organ transplant recipients, patients with acquired immune deficiency syndrome (AIDS) and other immuno-compromising conditions, low-birth-weight newborns, critically ill patients requiring multiple catheters or patients with cancer [4]. *Candida* species now rank as the fourth most common causes of nosocomial infections in

the United States and the assigned mortality rate is 35%. Approximately 70% of women experience vaginal infections caused by *Candida* species and 20% suffer recurrence after treatment [22]. Invasive fungal infections, such as candidosis, is a major public health problem and are primary fungal infections encountered in immunocompromised patients undergoing prolonged hospitalization [23]. *Candida* are the fourth leading cause of nosocomial bloodstream infection, with an attributable mortality rate of about 40% [4]. In the case of vaginal *Candida*, colonisation rates again vary with studied groups, with rates of 41% and 21% reportedly occurring in type 1 and type 2 diabetic patients, respectively [24]. Pregnant women are also reported to have a high incidence of vaginal candidal carriage [25], and vaginal candidosis is one of the most common superficial infections in reproductive age women [26].

Although most cases of candidosis have been attributed to *C. albicans*, more recently NCAC species have been identified as common pathogens. The prevalence of these species in human infection has been changing in recent years. In European countries, analysis has showed that the incidence rates of candidosis caused by NCAC species were 14% each for *C. glabrata* and *C. parapsilosis*, 7% for *C. tropicalis* and 2% for *C. krusei* [27]. *C. tropicalis* has emerged as the second or third most common agent of candidosis, mainly in nosocomial urinary tract infections [28]. *C. parapsilosis*, although generally regarded as one of the less virulent *Candida* species, is now attributed to infection following its transfer via the hands of healthcare workers [29]. *C. glabrata*, the newest recognised nosocomial *Candida* pathogen, has particular importance because of its naturally high resistance to certain antifungal agents, specifically the azoles [30].

Nowadays, several studies have reported enhanced *Candida* species epithelium invasion/colonisation and candidosis episodes caused by co-infection (two or more *Candida* species combined or associated to other species). Enhanced *C. albicans* colonisation and invasiveness of *C. glabrata* and led to increased damage in the reconstituted human oral epithelium [31]. In 2013, Kali et al. [32] showed that *Candida* species were emerging as a potentially pathogenic fungus in patients with broncho-pulmonary diseases, and demonstrated synergistic growth promoting association of *Candida* species and *Mycobacterium tuberculosis* in 40% of patients with pulmonary

tuberculosis. In 2010, Martins *et al.* [33] described *Candida* species oral co-infection episodes in patients attending a dental clinic in Braga, Portugal, where the combination of *C. albicans* - *C. glabrata* was the most commonly found, as noted in other studies [34–38].

Fungal endocarditis is an uncommon complication of invasive candidal infection, but there is an increasing incidence over the last 15 years [39,40]. Neonatal endocarditis caused by *Candida* is associated with high mortality and morbility [41]. Successful treatment often requires prolonged systemic antifungal therapy as well as surgical intervention [42]. Some cases of endocarditis had been identified in drug abusers due to co-infection between *C. albicans* and *C. tropicalis* [43]. In 2009, Daas *et al.* [44] reported an endocarditis episode caused by the combination of *C. parapsilosis* and *P. aeruginosa.*

1.1.3. Virulence Factors

Virulence factors are all aspects associated with metabolic pathways that directly interact with the host tissue causing damage and infection. There are several lines of argument for what is true virulence factors are generated by *Candida* species. Viruelence factors have been described as: “all traits required to establish disease” [45], “factors that interact directly with mammalian host cells” [46] and “a component of a pathogen that damages the host” [47]. In order to establish an infection, opportunistic pathogens have to evade the immune system, survive and divide in the host environment and spread to the new tissue.

Virulence factors of *Candida* species include the ability to: (I) undergo polymorphism, (II) adhere to biotic or abiotic surfaces, (III) secrete hydrolytic enzymes and (IV) form biofilms.

I- Polymorphism

A reversible morphologic transition (Figure 1.1) between unicellular yeast cells and filamentous phase (hyphae and pseudohyphae) is an important virulence factor of some *Candida* species as described above. This ability provides cells with the flexibility of being able to adapt to hostile conditions imposed by the human body [17]. The filamentous phase is constituted by a cell wall, which contains three times more chitin than the yeast

form. This composition increases its resistance to phagocytosis, gives more mechanical strength allowing enhanced epithelium colonisation and invasion into epithelial layers of tissues [17,48]. Furthermore, there is evidence that the yeast-hyphal switch is affected by temperature, pH (see section 1.2) and other virulence factors described in detail bellow. For instance, expression of secreted aspartyl proteinase (*SAPs4-6*) genes occur specifically during hyphal development [49]. Moreover, Hube et al., 2001 [50] showed that phospholipases D (*PLD1*) is necessary for yeast-to-hyphal transition by *Candida albicans*.

II- Adherence to biotic or abiotic surfaces

Adherence of *Candida* to host cells or abiotic surfaces is seen as an essential early step in the establishment of disease [10]. This adhesion is dependent on several factors such as the presence of cell wall proteins, that are recognized by receptors (*e.g.* fibronectin, fibrinogen and vitronectin) present on epithelial, endothelial and foreign-body surfaces, and cell surface physicochemical properties [51]. As surfaces of *Candida* and epithelial cells are generally negatively charged establishment of a successful adherence is, in part, dependent on the sum of non-specific factors contributing to the total free energy of interaction. These include attractive Lifshitz-van der Waals forces, hydrophobic interaction and Brownian movement, as well as the repulsive effects of the electrical double layer of cells [52].

Host cell recognition and colonisation by *Candida* species is facilitated by several adhesins (Table 1.1) that bind specifically to peptides or sugar residues of other cells and/or increase the cell surface hydrophobicity, promoting the binding to abiotic surfaces through hydrophobic interactions [53].

Adhesins on the cell surface of *C. albicans* can interact with serum proteins, components of the extracellular matrix (ECM) of host tissues and immobilised ligands such as cadherins or integrins [54]. An important serum component that *C. albicans* can bind to is Factor H (FH) [55] which is a key regulator of the alternative pathway (AP) of complement, and incorporation of FH on the surface of *C. albicans* prevents AP activation [56]. Laminin, fibronectin, collagen, entactin, vitronectin and tenascin are all ECM proteins that *C. albicans* can interact with [57].

Table 1.1 - Examples of *Candida albicans* adhesins and associated host cell ligands [58]

Candida adhesin	Host cell receptor
Integrin analog [59–61]	iC3b, Arginine-glycine-aspartic acid
Fibronectin adhesin [62–64]	Fibronectin and vitronectin receptors
Fucoside binding adhesin [65–67]	glycoside (glycoprotein or glycolipid) receptor
GlcNAc-binding protein [66,67]	N-Acetylglucosamine
Fimbrial adhesin [68]	βGalNAc(1-4β-Gal)
Hyphal Wall Protein 1 [69,70]	Covalently linked to cell wall β1,6-glucan through a remnant of its glycosylphosphatidylinositol (GPI) anchor
Agglutinin-like Sequence family [71–73]	Linked to β1,6-glucan and behave as a GPI module
Enhanced Adherence to Polystyrene [74]	GPI-attached site ω and a valine at the ω-5 site

Members of the *agglutinin-like sequence* (*ALS*) gene family of *C. albicans* encode for large cell-wall glycoproteins, some of which are implicated in the adhesion of the organism to host surfaces [75,76]. The *ALS* gene family comprises 8 members (*ALS1-7,9*) and all have a similar three-domain structure and are associated with the β-1,6 glucan of the cell wall of *C. albicans* [72]. *Hyphal wall protein* 1 (*HWP*) is another protein involved in *C. albicans* adhesion to epithelial cells and this protein is perhaps the most widely studied adhesin of *C. albicans* [77]. Glutamine residues in the N-terminal domain of *HWP1* can be cross-linked to unidentified host proteins by host transglutaminase activity and this leads to covalent attachment of the yeast to host epithelial cells. Recently, the gene encoding for *C. albicans EAP1* was identified. This gene was originally investigated because of its ability to encode for a protein mediating adhesion to polystyrene of a *Saccharomyces cerevisiae* flocculin-deficient strain. *EAP1* encodes for a glycosylphosphatidylinositol-anchored, glucan-cross-linked cell wall protein that has, since then, been shown to facilitate adhesion of *C. albicans* to epithelial cells as well as polystyrene [54]. After adherence to surfaces has been established, colonisation and growth of *C. albicans* is essential to sustain the presence of the organism at the host site.

The *in vitro* adherence of *C. glabrata* to epithelial cells is mediated by *EPA* genes, of which, up to 23 different genes have already identified, although the function of the majority is only partially understood [78]. There are few studies about Epa proteins, but it is already known that *EPA1* encodes a lectin that recognizes host N-acetyl lactosamine containing glycoconjugates and this confers adhesion of fungal cells to mammalian surfaces. There is relatively limited information about the adhesins of NCAC species. However, Panagoda *et al.,* 2001 [79] showed that the initial adhesion of *C. parapsilosis* was associated with surface hydrophobicity. The same study comparing *C. albicans* and *C. parapsilosis* adhesion reported a greater ability (21%) of *C. parapsilosis* to adhere to buccal epithelial cells with an increase of 14% in the extent of adhesion to acrylic. Looking to proteins from *C. tropicalis* cell wall, at least 3 *ALS* genes were identified through Southern and Western blotting analysis with anti-Als antibody [76]. However, no further work has been undertaken in this area.

III- Secretion of hydrolytic enzymes

The production and release of hydrolytic enzymes by extracellular secretion, are also key factors of *Candida* virulence [17,80]. These enzymes help mediate host adhesion, tissue invasion and destruction, and are also thought to be responsible for modulating host immune responses [17]. The most discussed hydrolytic enzymes released during the pathogenic process are secreted aspartic proteinases (*SAPs*), but phospholipases, hemolysins and lipases are also involved in *Candida* virulence [80].

The *SAP* family of *C. albicans* is currently known to comprise 10 genes encoding for proteinases with masses of 35 to 50 kDa. *SAPs1-3* and *SAPs4-6* are thought to represent two subfamilies [81]. Studies have demonstrated that *C. albicans*, *C. parapsilosis* and *C. tropicalis* are good producers of Saps [10,82,83]. Gene expression of *SAPs1-6*, appears to be related to adherence, tissue damage, and changes in the immune response [81,84,85]. *SAPs4-6* are expressed by *C. albicans* during hyphal invasion of a reconstituted human oral epithelium, oral infection [86] and are linked with hyphal formation and invasion of the epithelium [81] and apoptosis of epithelial cells [87]. In the case of *C. parapsilosis*, three genes have been identified (*SAPP1-3*), but two of these

remain uncharacterized. In addition, *C. tropicalis SAPs* are encoded by four genes (*SAPT1-4*), but only *SAPT1* has been characterized. Only one study has been done that demonstrates an ability of *C. glabrata* to produces proteinase, but the proteinase type remain unknown [10].

In addition to *SAPs*, *C. albicans* has two other hydrolytic gene families, namely the lipases (*LIP*) and phospholipases (*PL*), and these are associated with candidal adhesion, nutrient acquisition and invasion of epithelial surfaces [88].

The *LIP* gene family of *C. albicans* comprises of at least 10 genes (*LIP1-10*) [89], whilst 7 phospholipase genes of *C. albicans* have been reported (*PLA*, *PLB1-2*, *PLC1-3*, and *PLD1*) [90]. *LIPs* are involved in the hydrolysis and synthesis of triacylglycerols and are characterised by having stability at high temperatures and in organic solvents, and being resistant to proteolysis [91]. It is important to emphasize that the role of *PLs* is to hydrolyse ester bonds in glycerophospholipids and this causes host cell membrane damage, facilitating invasion of host tissues by the exposure of receptors to adhesions. *PLB1* is proposed to have an important role in the secretion of phospholipase B by *C. albicans* [83]. NCAC species also produce extracellular *PLs*, but in lower amounts than *C. albicans* [10].

Hemolysins are enzymes that induce the rupture of hemoglobin, leading to release of elemental iron from host cells [10,92]. Although essential for growth of *Candida*, free iron sources are very limited in the body as most iron is appropriated by host proteins, especially transferrin [93]. Thus, hemolysins secreted by *Candida* serve to replenish the organisms' supply of iron from hemoglobin. A variety of *Candida* species produce α or β hemolysin, with *C. albicans* and *C. dubliniensis* being the greatest producers, and production may increase in the presence of elevated blood glucose concentrations [94–96]. The putative gene involved in expression of *Candida* hemolysin is *HLP* (*Haemolysin Like Protein*) [10,92].

IV- Candida Biofilms

One of the major contributions to *Candida* species virulence is its versatility to adapt to a variety of different habitats and the formation of surface-attached microbial communities known as biofilms (Figure 1.4) [97,98].

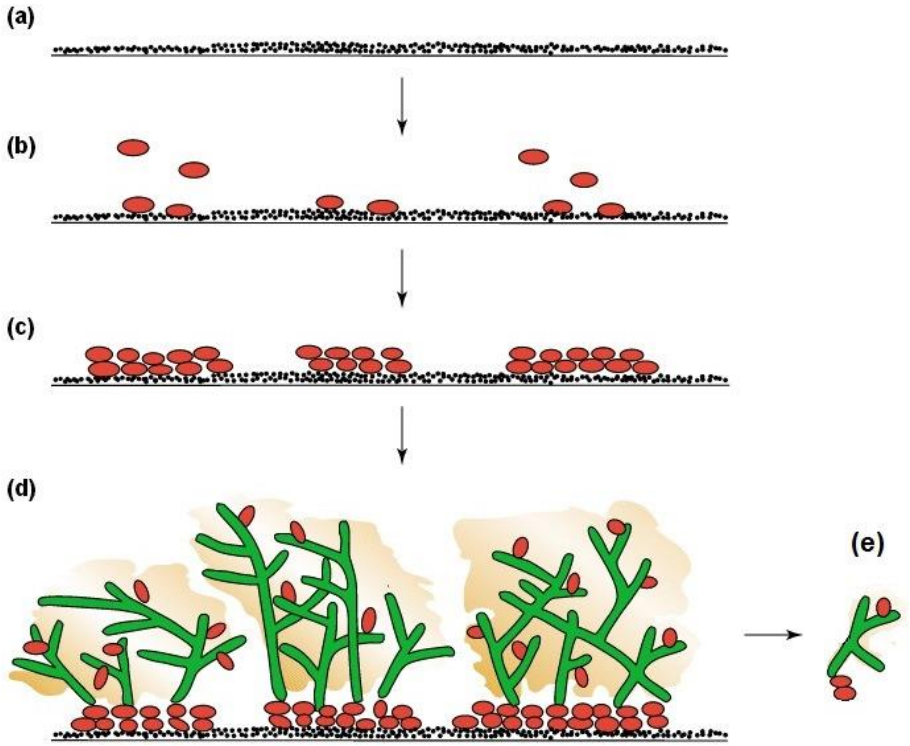

Figure 1.4 - Schematic of biofilm formation on a polyvinylchloride catheter surface. **(a)** Catheter surface with an adsorbed conditioning film of host proteins (black dots). **(b)** Initial yeast cell (red) adhesion to surface. **(c)** formation of basal microcolony layers of microorganisms. These anchor each microcolony to the surface. **(d)** completion of microcolony formation by addition of the upper, mainly hyphal layer and matrix material (yellow) that surrounds both yeasts (red) and hyphae (green). Mature biofilms contain numerous microcolonies with interspersed water channels to allow circulation of nutrients. **(e)** Cells released or detached from the biofilm spread into the environment, dispersing infections and forming new biofilms. Adapted from [98].

Biofilm formation (Figure 1.4) begins with the attachment of free-floating cells to a surface. These first cells initially adhere weakly and reversibly to the surface via Van der Waals forces. Some microorganisms are not able to attach to a surface on their own, but are able to anchor themselves to the matrix or directly to other earlier colonising

microorganisms. Once colonisation has begun, the biofilm grows through a combination of cell division and recruitment. In the maturation step, the biofilm is established and may change only in shape and size. The final step is dispersion, where cells detach from the biofilm and start to spread and colonise new surfaces.

In most natural environments, microorganisms exist predominantly as biofilms rather than as planktonic or free-floating cells. Biofilms represent structured microbial communities that are attached to a surface, where the individual microorganisms are embedded within a matrix of extracellular polymeric substances (EPS) [99].

Generally, in addition to water, the composition of EPS includes, carbohydrates, proteins, minerals, hexosamines and extracellular DNA. However, a large portion of the biofilm matrix still remains to be identified [100].

Microorganisms that live in a biofilm have significantly different properties than free-floating (planktonic) microorganisms, as the presence of the matrix, protects embedded biofilm cells. In addition, the microenvironment formed may allows the establishment of symbiotic microbial associations that can provide essential nutrients to some species. The close contact between cells of different species/strains favors the transmission of mobile genetic elements [101].

Both commensal and pathogenic organisms can form biofilms in human tissues and around 60-65% of microbial infections are thought to have a biofilm origin [102]. Although multi-species biofilms are most commonly encountered, single-species biofilms are also evident in some infectious diseases [103].

Microbial biofilms are notoriously resistant to a variety of antimicrobial agents, including antibiotics/antifungal, antiseptics, host defence molecules and industrial biocides. Susceptibility studies have revealed that biofilms formed by *C. albicans* may be up to 100-fold more resistant to antifungal drugs than planktonic cells [100,104,105].

Candida species can adhere and form biofilms on natural host surfaces or on biomaterials of medical devices [106]. The overall organization of a *Candida* biofilm is generally similar to a bacterial one, but its structural details are highly dependent upon the conditions under which the biofilm formed. Numerous experimental parameters, such as the surface material, medium and conditions of incubation will influence the biofilm

structure and the morphology of its cells [107]. The formation of *Candida* biofilms has important clinical repercussions because it provides a considerable resistance to antifungal therapy by limiting the penetration of these substances through the matrix and the protection afforded against host immune defenses [99,108].

1.1.4. Antifungal Resistance

Since their discovery during the 20th century, antimicrobial agents have substantially reduced the risk posed by infectious diseases. The use of these antimicrobials, combined with improvement in nutrition and the implementation immunization programs, has led to a notable drop in deaths from infectious diseases. Although antimicrobials have saved the lives of millions of people, in the recent years the advantages of these drugs has been compromised with the appearance of resistant microorganisms. When infections are caused by resistant microorganisms, treatment has to be switched to second- or third-line drugs, which are usually more expensive and occasionally more toxic.

Candidosis can be treated using several classes of drug (azoles, candins or polyenes). The choice of the agent depends on the local epidemiology and the origin of the infection [109]. The cost of a candidosis episode has been estimated at $44 000 for adults and $28 000 for neonates [110,111]. However, a four-fold increase in hospital spend on antifungals has been recognised, which is not accounted for solely by increases in the number of fungal infections. An abrupt change in the way drugs are prescribed and the use of newer antifungal drugs, sometimes in combination therapies, are part of the problem. At least 70% of the antifungal drugs prescribed are part of an empirical strategy [112]. In addition, the emergence of NCAC species in infection has been an issue as these tend to be inherently less susceptible to the available antifungal drugs like the azoles and their derivatives, which are frequently the antifungal agents of choice against candidosis [113,114]. For example, since the introduction of fluconazole in 1990 for the treatment of candidosis, empirical antifungal therapy has been driven by fear of infections caused by *C. glabrata* as this species exhibits reduced susceptibility to fluconazole and cross-resistance to other azoles [115].

Increased resistance to antifungal agents is one of the defining characteristics of biofilms [116]. Over the recent years, several factors that play a role in fungal biofilm resistance have been described, including the altered physiological state, cell density, extracellular polymeric substances, efflux pump-mediated resistance, overexpression of drug targets and the presence of persister cells [17,116]. In biofilms, nutrient and oxygen limitation are responsible for slowing down the growth rate and activity of cells embedded in the biofilm. This effect would render agents active against the cell division process less effective and also result in a slower or inefficient drug uptake [98,99,117]. Furthermore, other factors like pH, temperature, changes in osmolarity and ionic stress can also effect conserved signaling pathways and perhaps alter antifungal susceptibility, which suggests that more complex factors may be involved in drug resistance of biofilms [118,119]. For example, *C. albicans* biofilms show an increased resistance to a wide range of conventional antifungal agents, such as amphotericin B and fluconazole. The biofilm phenotype can be about twenty times more resistant to amphotericin B and one hundred times more resistant to fluconazole compared with the planktonic growth form [105,120].
Gathering together the inherent resistance of *Candida* species and the difficulty in biofilm treatment, there is a clear necessity to develop new alternative antifungal agents, in order to increase the spectrum of activity against *Candida* species. To partly address this, there has recently been an increased interest in natural phytochemicals derived from fruits, vegetables, plants and herbs that have been reported to possess a wide range of biological effects, including antioxidant, antimicrobial and anti-inflammatory actions [121]. Essential oils and their main components have many applications in popular medicine, food, beverages, preservation, cosmetics as well as in the fragrance and pharmaceutical industries [122]. Essential oils are mixtures of compounds obtained from spices, aromatic herbs, fruits, and flowers and characterised by their aroma [123]. The antimicrobial properties of essential oils have been known for a long time, and various research has been conducted into their antimicrobial activities against various bacteria and fungi [124]. Furthermore, in recent years, interest in phenolic compounds has also raised. Such compounds include phenolic acids and flavonoids that have been reported to inhibit various pathogenic bacteria and fungi [125–127]. In 2011, Barros *et al*. [128] highlighted

the importance of wild plants as sources of phenolic compounds, such as phenolic acids, flavonoids and anthocyanins and several studies have reported the effectiveness of these phenolic compounds in the inhibition of various pathogenic bacteria and fungi [125,126,129–131].

1.2. Environmental factors

Effective responses to environmental changes are fundamental for the survival of microorganisms. Environmental adaptation is particularly relevant for pathogens, which must counteract the defence systems of their host as well as tune their metabolism and stress homeostatic mechanisms to the complex microenvironments they encounter. Different environmental factors, such as temperature, pH, glucose, hormones, can influence the normal growth, infection and pathogenicity of *Candida* species [105,132,133].

Although the parameters that triggers morphologic *Candida* dimorphism are not yet well understood, *in vitro* studies have shown that this behaviour is influenced by temperature and pH. Unicellular yeast phase growth is stimulated at 25°C and acidic pH, whereas filamentous growth is favoured at 37°C and with a neutral pH [84]. These changes are often mediated by cell surface receptors that initiate signal transduction cascades resulting in altered activity of transcription factors and thus modification of gene expression. Variation in pH can also influence *Candida* gene expression. In 2001, Lane *et al*. [134] showed that altered pH effects expression of virulence factors including the *SAPs*. Glucose plays a central role as a carbon and energy source and glucose sensing and response is therefore highly involved and closely regulated in most organisms. The importance of glucose to yeast cells is highlighted by the large number of hexose transporters they possess. *Saccharomyces cerevisiae* has at least 17 different hexose transporters that are expressed under different conditions, whilst *C. albicans* appears to have over 20 [135].

In 2012, Silva *et al.* [10] showed that biofilms were readily formed by *C. parapsilosis* grown in media containing higher glucose. Furthermore, the production of *Candida* haemolytic factor may be regulated by the presence of glucose in the growth medium.

As stated above, vaginal candidosis (VC) is an opportunistic infection caused by *Candida* species that affects healthy women. Several episodes of VC frequently occur during pregnancy and the luteal phase of the menstrual cycle, when the levels of progesterone and estrogen are elevated [136]. The same study [136] showed that progesterone has no effect on VC in mice, but an excess of estrogen promoted *Candida* growth. They also found that the excess estrogen reduces the ability of skin cells to prevent colonisation of *Candida* on the vaginal walls. Furthermore, it is known that in the absence of estrogen treatments, VC is short-lived, with a low fungal burden in the vagina [137].

1.3. Reconstituted human epithelium

Oral and vaginal epithelia are complex multi-layered, multi-cellular tissues consisting of increasingly differentiated epithelial cells. As a consequence the use of monolayer cultures *in vitro* infection studies are unlikely to be representative [138,139].

Several model systems have been used to study *Candida*-host interactions. Murine models and *in vitro* monolayer cultures have generated some data on the likely virulence and host factors that contribute to the development of candidosis [140]. Murine models of oral candidosis have been used to identify several potential virulence factors that may play a role in pathogenesis [141,142], but since *C. albicans* does not naturally colonise the oral cavity of mice it is not clear how representative these studies are. Furthermore, mouse models do not completely mimic human candidosis as there are significant differences between the immune systems of humans and mice [143], which may make results difficult to interpret. There are also ethical restraints relating to experiments involving animals.

Recently multi-layered organotypic three-dimensional *in vitro* culture systems have been developed to mimic various epithelia. The *in vitro* reconstructed human epidermis (SkinEthic Laboratories, Nice, France) consists of normal human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface, in a chemically defined medium. These models are histologically similar to those of the *in vivo* human epidermis but do lack a keratinised surface layer and stratum corneum (Figure 1.5).

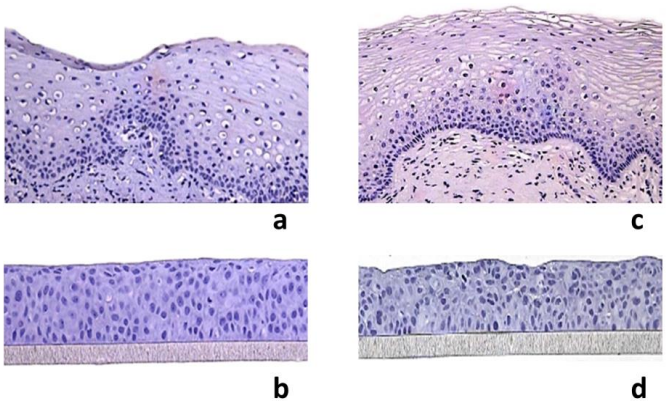

Figure 1.5 - SkinEthic Human Epithelium (RHE). **(a)** oral mucosa epithelium *in vivo*, **(b)** reconstituted human oral epithelium *in vitro*, **(c)** vaginal mucosa epithelium *in vivo* and **(d)** reconstituted human vaginal epithelium *in vitro.* Adapted from [144,145].

The reconstituted human oral epithelium (RHOE) (Figure 1.5 b) and reconstituted human vaginal epithelium (RHVE) (Figure 1.5 d), based upon the TR146 buccal carcinoma cell line and the A431 vaginal squamous cell carcinoma cell line, respectively, are effective for modeling the initial interactions of *Candida* species with human cells [31,146–149]. These models require at least 3 days to be established and can be maintained thereafter for 2-4 days. In these models, experiments of infection are highly reproducible [31,147,150]. These models can be used for the direct analysis of pathogen-epithelial cell interactions, the evaluation of the host immune response, to investigate cellular interactions or protein and gene expression. It is also possible to study the impact of innate immunity or the antifungal activity of natural and non-natural compounds [148].

1.4. References

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Chapter II

Anti-candidal activity of Natural extracts/compounds from wild flowers used in Portuguese folk medicine

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Abstract

Life-threatening mycoses caused by opportunistic fungal pathogens (mainly species from the genus *Candida*) are frequently encountered as nosocomial infections, and currently represent a major health challenge. Given the relatively limited number of available antifungal agents and the increasingly apparent resistance evident with those that are available, it is essential to identify new antifungal compounds. Of particular interest are those derived from natural sources and these agents frequently exhibit a wide activity spectrum against *Candida* species with little resistance. In this chapter, the antifungal potential of natural plants extracts (*Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* and *Cistus ladanifer*) and of four phenolic compounds (gallic acid, catechin, luteolin and quercetin) was screened. All of these agents originate from wild plants located in North Eastern Portugal and tests were undertaken using planktonic cells and biofilms of *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 2001, *Candida parapsilosis* ATCC 22019 and *Candida tropicalis* ATCC 750.

The minimal inhibitory concentrations (MIC) of plant extracts and phenolic compounds were determined against *Candida* planktonic cells and the effect of phenolic compounds on *Candida* biofilms was assessed by quantification of colony forming units (CFUs).

Overall, the highest antifungal effect against all the *Candida* species was showed by the extract from *Rosa micrantha* (MIC ≤ 0.156 mg/ml). Results using phenolic compounds showed that gallic acid had the highest mg/ml effect against the four *Candida* species in planktonic form (MIC ≤ 0.156 mg/ml). Catechin showed a similar effect against *C. albicans* cells. Additionally, gallic acid and quercetin demonstrated only a slight effect against *Candida* species biofilms.

2.1. Introduction

The genus *Candida* is composed of an extremely heterogeneous group of over 150 species [1], and it is well established that a minority of these are implicated in human candidosis [1,2].

A major virulence factor of *Candida* is its ability to adapt to a variety of different habitats and the subsequent formation of surface-attached microbial communities known as biofilms [3–5]. *Candida* yeast within a biofilm can have significantly different properties from 'free-floating' or planktonic microorganisms, due to the existence of extracellular polymeric substances (EPS) surrounding them. The physical structure and chemical composition of a biofilm is largely determined by the EPS and allows different microorganisms to cooperate and interact in various ways. Importantly, the presence of EPS confers a certain degree of protection against host defence molecules and cells as well as administered antimicrobial agents. Of note is that candidal biofilm communities reportedly present elevated tolerance to typical antifungal drugs, like amphotericin B and fluconazole [6,7].

Biofilms can be found on different surfaces including those that are biotic (mucosal surfaces) and abiotic (invasive medical devices) [8,9]. The biomedical significance of these biofilms is considerable, as most hospital infections have origins within preformed biofilms [10,11].

Currently, it is widely acknowledged that *Candida albicans* and, recently, non-*Candida albicans Candida* (NCAC) species exhibit high levels of resistance to antifungal therapies such as the azole drugs and their derivatives, which continue to be the empirical treatment of choice for *Candida*-related infections [12–15]. In addition candidosis can be treated with echinocandins and polyene antifungal classes. The selection of the antifungal agent depends on the epidemiology local, the percentage of resistant strains to fluconazole and even the origin of the infection [16]. Additionally, at least 70% of the antifungal drugs are prescribed empirically [17] and consequently it has been noted a decreased in susceptibility to fluconazole and cross-resistance to other azoles.

Thus, in order to overcome this clinical problem, an enlarged interest to find new effective natural drugs, such as plant extracts compounds (specifically some phenolic compounds) and essential oils, has been observed [18–20].

In this context, the main objective of the present work was to evaluate the antifungal potential effect of four plant extracts from the North Eastern of Portugal, *Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* and *Cistus ladanifer*, and four phenolic compounds, gallic acid, catechin, luteolin and quercetin identified in those plants against *Candida* planktonic and biofilm cells (*C. albicans* ATCC 90028, *C. glabrata* ATCC 2001, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 750).

2.2. Material and methods

2.2.1. Preparation of plant extracts

Plants extracts were collected from *Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* [21] (flowers extracts) and *Cistus ladanifer* [22] (fresh leaves extract) originating in the Natural Park of Montesinho territory, Trás-os-Montes, North Eastern Portugal. Extracts were obtained according to local medicinal criteria of use and each plant growth pattern. Extracts were lyophilized (Ly-8-FM-ULE, Netherlands) and stored at -20°C for subsequent analysis. The procedure described below was done by the colleagues from CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Bragança, Portugal [21,22]. Briefly, extracts were obtained by treating each plant sample (1 g) with 30 ml of methanol:water 80:20 (v:v) at room temperature, 150 rev/min, for 1 h. The extract was filtered through Whatman number 4 paper. The residue was then re-extracted twice, with additional 30 ml portions of methanol:water 80:20 (v:v). The combined extracts were evaporated at 35°C (Rotary Evaporator Büchi R-210) to remove methanol. The aqueous phase was lyophilized and re-dissolved in distilled water at 200 mg/ml for antifungal assays or in 20% aqueous methanol at 5 mg/ml and filtered through a 0.22 μm disposable LC filter disk for High Performance Liquid Chromatography (HPLC) analysis.

2.2.2. Preparation of phenolic compounds

The extraction, identification and quantification of phenolic compounds from flowers of *Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* [21], *Cytisus multiflorus* [23] and fresh leaves of *Cistus ladanifer* [22] was previously described by Barros *el al.* [21–23] using HPLC-DAD/ESI-MS. Eight different phenolic compounds were highlighted as being the main compounds; three phenolic acids: caffeic acid (Caf), ellagic acid (Ell), gallic acid (Ga); and five flavonoids: apigenin (Api), catechin (Ca), kaempferol (Kae), luteolin (Lu) and quercetin (Quer).

2.2.3. *Candida* strains

Four *Candida* reference strains from the American Type Culture Collection (ATCC), namely *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 2001), *Candida parapsilosis* (ATCC 22019) and *Candida tropicalis* (ATCC 750) were used in this study.

2.2.4. Minimal inhibitory concentration determination

Prior to undertaking antifungal assessment experiments for the plant extracts/phenolic compounds activity experiment, all reference species were cultured on Sabouraud Dextrose Agar (SDA; Merck, Germany) for 24 h at 37°C.

For antifungal assessment of plant extracts, at least five colonies (1 mm diameter) of each *Candida* species were suspended in 5 ml of sterile saline solution (0.85% of NaCl in water) and the resulting yeast suspension vortex mixed for 15 s. The suspensions were then adjusted by spectrophotometric method, adding saline solution to reach the value of 0.5 in the McFarland scale corresponding to a final concentration of $3.0 \pm 2.0 \times 10^6$ cells/ml.

In the case of antifungal phenolic compounds activity, *Candida* were cultured in Sabouraud Dextrose Broth (SDB; Merck, Germany) for 18 h at 37°C under agitation at 120 rev/min. After incubation, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice using 15 ml of phosphate buffer saline (PBS; pH 7, 0.1 M). Pellets formed were suspended in 10 ml RPMI 1640 medium (Sigma, St Louis, USA), buffered to pH 7 and the cellular density adjusted to $2x10^7$ cells/ml using a Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany). It should be pointed out that this cell concentration was different from the one used with the extracts, since it was intended to test this compounds also against biofilms, and therefore, cell inoculum concentration was adjusted.

The Minimum Inhibitory Concentration (MIC) of the agents against all the species under study, was determined according to the guidelines described by the National Committee for Clinical Laboratory Standards (NCCLS) [24], with some modifications. Initially, serial dilutions of each plant extract and phenolic compound were prepared in RPMI 1640 medium to provide concentrations ranging from 0.05 to 5 mg/ml and 0.156

to 1.25 mg/ml, respectively. These concentrations were stored at -20°C until used in the assay. Volumes of each plant extract/phenolic compound (100 µl), at a two-fold final concentration together with 100 µl of *Candida* suspension were mixed in the wells of microtitre plate (Orange Scientific, Braine-l' Alleud, Belgium). The 96-well plates were incubated at 37°C for 48 h and the MIC determined by direct observation and by determination of the number of colony forming units (CFUs). CFUs were determined after appropriate serial dilutions of the well contents in PBS and by plating 10 µl of each cell dilution on to SDA. After 24 h incubation at 37°C, the number of the colonies was counted. Experiments were performed on three separate occasions and in each experiment at least triplicate samples were tested. Yeast culture without test agent and RPMI without *Candida* were included as controls.

2.2.5. Antibiofilm activity of phenolic compounds on *Candida* biofilms

Standardised *Candida* cell suspensions (200 µl containing $1x10^7$ cells/ml in RPMI 1640 medium) were placed into the wells of 96-well microtitre plates (Orange Scientific, Braine-l`Alleud, Belgium) and incubated at 37°C on a shaker at 120 rev/min for 24 h. Negative controls (200 µl of RPMI 1640 medium devoid of *Candida*) were also included. After 24 h, the culture medium was aspirated and non-adherent cells removed by washing the biofilms once in 200 µl of PBS. Then, 200 µl of each phenolic compound (prepared in RPMI 1640 medium; concentration range 0.625 to 5 mg/ml) was added to each well. The biofilms were incubated for further 24 h at 37°C on a shaker at 120 rev/min. The effect of the phenolic compounds on *Candida* biofilms was then assessed by quantification of the number of CFUs. To determine this, culture medium containing test agent was removed and the biofilms washed once with 200 µl of PBS.

The biofilms resuspended in 200 µl of PBS were removed from the wells by physically scraping the respective wells. The recovered biofilm suspension was vigorously vortexed for approximately 2 min to disaggregate cells. Serial dilutions were then prepared in PBS, and these were cultured on SDA for 24 h at 37°C. Experiments were performed in triplicate and in at least three independent assays.

2.3. Results and Discussion

2.3.1. Antifungal activity of the plant extracts

In recent years, an increasing number of *Candida* species that are resistant to the commonly used antifungal agents has become evident [25]. In order to overcome this problem, the identification of new antifungal compounds is highly important. Particular focus has been directed to naturally derived compounds such as those evident in plant extracts. Often, these agents exhibit antimicrobial effects against a wide range of microorganisms with little or no resistance being evident.

In this study, the aim was to examine for the first time, the antifungal effect of extracted compounds from the Portuguese indigenous plants *Castanea sativa*, *Filipendula ulmaria*, *Rosa micrantha* (flowers extracts) and *Cistus ladanifer* (fresh leaves extract). The minimum inhibitory concentration (MIC) values against four different species ranged from 0.05 to 0.625 mg/ml depending on agent were present in Table 2.1. Furthermore, it is important to highlight that the extracts presented different activities against the different *Candida* species under study.

Table 2.1 – Minimum inhibitory concentrations (MIC; mg/ml) of wild plant extracts against *Candida* species

	MIC (mg/ml)			
	C. albicans ATCC 90028	*C. glabrata* ATCC 2001	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750
Castanea sativa	0.625	< 0.05	< 0.05	0.625
Filipendula ulmaria	0.625	< 0.05	< 0.05	0.156
Rosa micrantha	0.05	< 0.05	< 0.05	0.156
Cistus ladanifer	< 0.05	< 0.05	< 0.05	0.625

In 2001, Aligiannis *et al.* [26] proposed a classification for plant extracts, based on the MIC values obtained for *C. albicans* and this defined strong inhibitors (MIC up to 0.5 mg/ml), moderate inhibitors (MIC between 0.6 and 1.5 mg/ml) and weak inhibitors (MIC

above 1.6 mg/ml). According to this classification and the values present in Table 2.1, the extract of *C. sativa* extract presented moderate activity (MIC = 0.625 mg/ml) against *C. albicans* ATCC 90028 and *C. tropicalis* ATCC 750, but a strong activity (MIC < 0.05 mg/ml) against *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019. *F. ulmaria* extract showed similar behaviour, except in its activity against C. *tropicalis* ATCC 750, where it demonstrated strong activity (MIC = 0.156 mg/ml). In the case of *R. micrantha* extract, a different profile was found, as it showed a strong effect against the four strains assayed. This higher activity of *R. micrantha* extract could explain its effective and traditional use to treat acne and skin conditions caused by organisms closely related with *Candida* species [27]. The fresh leaves extract of *C. ladanifer* was a strong inhibitor of *C. albicans* ATCC 90028, *C. glabrata* ATCC 2001 and *C. parapsilosis* ATCC 22019 growth (MIC < 0.05 mg/ml), and a moderate inhibitor of *C. tropicalis* ATCC 750 growth (MIC = 0.625 mg/ml). Bruni *et al*. [28] also demonstrated that *C. albicans* ATCC 48274 were markedly inhibited by oils rich in phenolics, aldehydes and alcohols. In fact, the results of chemical characterization performed by Barros *et al*. [22] showed that the plant extract of *C. ladanifer* was mostly derived from its phenolic compounds. This feature could be an explanation for the strong antifungal activity caused by *C. ladanifer* extract against the *Candida* species under study.

Additionally, the effect of the extracts on *Candida* species viability was assessed by measuring the number of colony forming units (CFUs). CFU enumeration (Figure 2.1) showed that antifungal activity was dependent on the test species. This evaluation is essential to differentiate fungicidal and fungistatic activity. The results showed that all the extracts yielded strong activity against the four *Candida* species studied (*P<0.001)*.

It should be noted that all the extracts caused at least a 2 Log reduction in CFUs for all strains, when tested at the lowest concentration (0.05 mg/ml). *Candida glabrata* ATCC 2001 was the strain that appeared to be most susceptible of those tested, with > 3 Log reduction in CFUs (Figure 2.1 b).

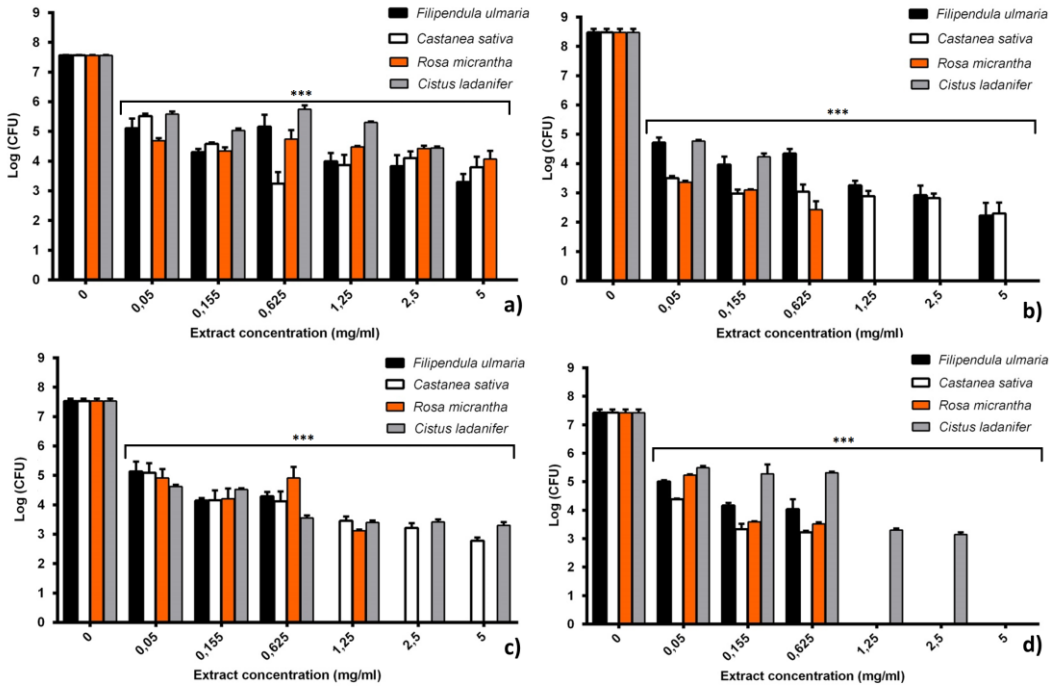

Figure 2.1 – Logarithm of number cells of *C. albicans* ATCC 90028 **(a)**, *C. glabrata* ATCC 2001 **(b)**, *C. parapsilosis* ATCC 22019 **(c)** and *C. tropicalis* ATCC 750 **(d)** cultured in different concentrations of four plant extracts, formed in SDB after 48 h. Error bars represent standard deviation. Concentrations that are significantly different (*** P<0.001) of each compound compared to control.

At concentrations of 1.25 mg/ml and 0.625 mg/ml respectively, only *R. micrantha* and *C. ladanifer* extracts were able to completely eliminate all *C. glabrata* ATCC 2001 cells (Figure 2.1 b). These results confirm the fungicidal effect of these plant extracts against *C. glabrata* ATCC 2001, compared with the fungistatic effect of the other two extracts. *F. ulmaria* and *R. micrantha* extracts caused similar effects against *C. parapsilosis*. However, *C. sativa* extract was not total inhibitory of *C. parapsilosis*, even when tested at its highest concentrations, and *R. micrantha* extract only produced total CFUs reduction at concentrations >2.5 mg/ml (Figure 2.1 c). *C. tropicalis* ATCC 22019 was highly susceptibility to *C. sativa*, *F. ulmaria* and *R. micrantha* (flower extracts), and importantly at concentrations >1.25 mg/ml, all these assayed extracts were able to cause total inhibition of the growth of this fungal strain (Figure 2.1 d). It is known that *C. tropicalis* is often

associated with urinary tract infections [29] and extracts of *F. ulmaria* are traditionally used to treat these infections [30]. Interestingly, despite *C. tropicalis* being tested species with highest genetic similarity to *C. albicans* [31], relative sensitivities of these species to all assayed extracts varied markedly. *C. albicans* was deemed to be most tolerant to these tested agents with none of the flower extracts able to give total inhibition of this species. However, the fresh leaves extract of *C. ladanifer* was able to completely reduce *C. albicans* growth at its highest concentration (5 mg/ml) tested. No studies concerning the antifungal activity of phenolic extracts from *C. ladanifer* are currently evident in the literature. However, Barrajón-Catalán *et al.* [32] reported on the antibacterial activity of a *C. ladanifer* aqueous extract against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) and deemed it more active against Gram-positive bacteria.

2.3.2. Antifungal activity of the phenolic compounds

Phenolic compounds are involved in plant growth and reproduction, and also can provide resistance against plant pathogens and even predators, protecting crops and seed from diseases [33,34]. Over 9000 natural antimicrobials have been identified, and these can exhibit anti-carcinogenic, anti-inflammatory, anti-oxidant [35] along with antibacterial and antifungal effects. The compounds isolated from each extract are presented in Table 2.2.

Table 2.2 – Presence or absence of each phenolic compound in the studied extracts (*F. ulmaria*, *C. sativa*, *R. micrantha*, *C. multiflorus* and *C. ladanifer*)

Natural Compound	Plant extract				
	Filipendula ulmaria [21]	*Castanea sativa* [21]	*Rosa micrantha* [21]	*Cytisus multiflorus* [23]	*Cistus ladanifer* [22]
Caffeic acid	+	-	-	-	-
Ellagic acid	-	-	-	-	+
Gallic acid	+	+	-	-	-
Apigenin	-	-	-	+	+
Catechin	-	+	+	-	-
Kaempferol	-	+	+	+	+
Luteolin	-	-	-	+	-
Quercetin	+	+	+	+	+

(+), presence; (-), absence of respective natural compound

In order to select the most likely antifungal compounds to be further examined, a disc diffusion method was performed according to the guidelines in NCCLS, M27-A2 document [24], with some modifications using the least and the most affected *Candida* species by the extracts (*C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001, respectively) and are presented in Table 2.3.

Table 2.3 – Efficacy of phenolic compounds (1 mg/ml) identified in wild plants from North Eastern Portugal *Candida* species

	C. albicans ATCC 90028	*C. glabrata* ATCC 2001
Caffeic acid	+	-
Ellagic acid	-	-
Gallic acid	+	+
Apigenin	-	+
Catechin	+	+
Kaempferol	-	+
Luteolin	-	-
Quercetin	-	-

(-), absence of halo; (+), presence of halo (≈3-6mm)

The results presented in Table 2.3, clearly demonstrate that gallic acid and catechin possessed antifungal activity against the *Candida* species tested. Hong *et al*. [36] confirmed that gallic acid a bioactive compound capable of inhibiting and killing *C. albicans* cells. Three phenolic compounds (ellagic acid, luteolin and quercetin) were not found to exhibit antifungal activity. From the evaluated compounds, gallic acid (phenolic acid), catechin (flavan-3-ols), luteolin (flavone) and quercetin (flavonol) [21–23], were chosen, for subsequent assays, due to their chemical characteristics and by the presence and absence of antifungal activity in the halo test. The MIC values of these four phenolic compounds were determined and ranged from 0.156 to 1.250 mg/ml (Table 2.4). Additionally, the MIC values were also confirmed determining *Candida* planktonic cells viability, by CFUs determination (Figure 2.2).

Table 2.4 – Minimum inhibitory concentration (MIC; mg/ml) values obtained with gallic acid, catechin, luteolin and quercetin against *Candida* species

Compounds	MIC (mg/ml)			
	C. albicans ATCC 90028	*C. glabrata* ATCC 2001	*C. parapsilosis* ATCC 22019	*C. tropicalis* ATCC 750
Gallic acid	< 0.156	< 0.156	< 0.156	< 0.156
Catechin	< 0.156	0.625	0.625	1.250
Luteolin	0.625	0.625	0.625	1.250
Quercetin	0.625	1.250	1.250	1.250

The results presented in Table 2.4 clearly demonstrated, as expected from the previous evaluation (Table 2.3), that gallic acid had highest (MIC < 0.156 mg/ml) antifungal activity against planktonic *Candida* cells. Even though, the actual mechanism of action of gallic acid on yeast cells has not been widely studied. Hong *et al*. [36] proved that gallic acid present in hydrolysable tannin extracted from barks of *Rhizophora apiculata* possessed anti-*C. albicans* activity. Additionally, catechin presented a similar effect against *C. albicans* ATCC 90028. It is important to highlight that the catechin, exhibited higher activity than reported by Haghighi *et al*. [37], who found an MIC value of 9.47 mg/ml against *C. albicans*. Although, luteolin has been previously reported to exhibit antimicrobial activity against *Bacillus cereus and Salmonella enteritidis* [38] and quercetin *against Staphylococcus aureus, Escherichia coli* and *Pseudomonas fluorescens* [39], in this present study, these phenolic compounds demonstrated a lower activity against all *Candida* species (≥ 0.625 mg/ml). The high resistance (MIC=1.250 mg/ml) of *C. tropicalis* ATCC 750 to all the tested flavonoids, with exception of gallic acid, should be noted.

In accordance with MICs values that we have obtained for traditional antifungal agents (*C. glabrata* ATCC 2001 0.625-1.250 mg/ml of fluconazole [40]) we consider the MICs values acceptable to explore as potential future candidates in the treatment of candidosis. Furthermore, many studies have focused on natural compounds and plant-derived active principles as possible alternative treatments against *Candida* infections [41–43].

Measuring *Candida* viability (CFUs determination) (Figure 2.2) is important when distinguishing between fungicidal and fungistatic effects.

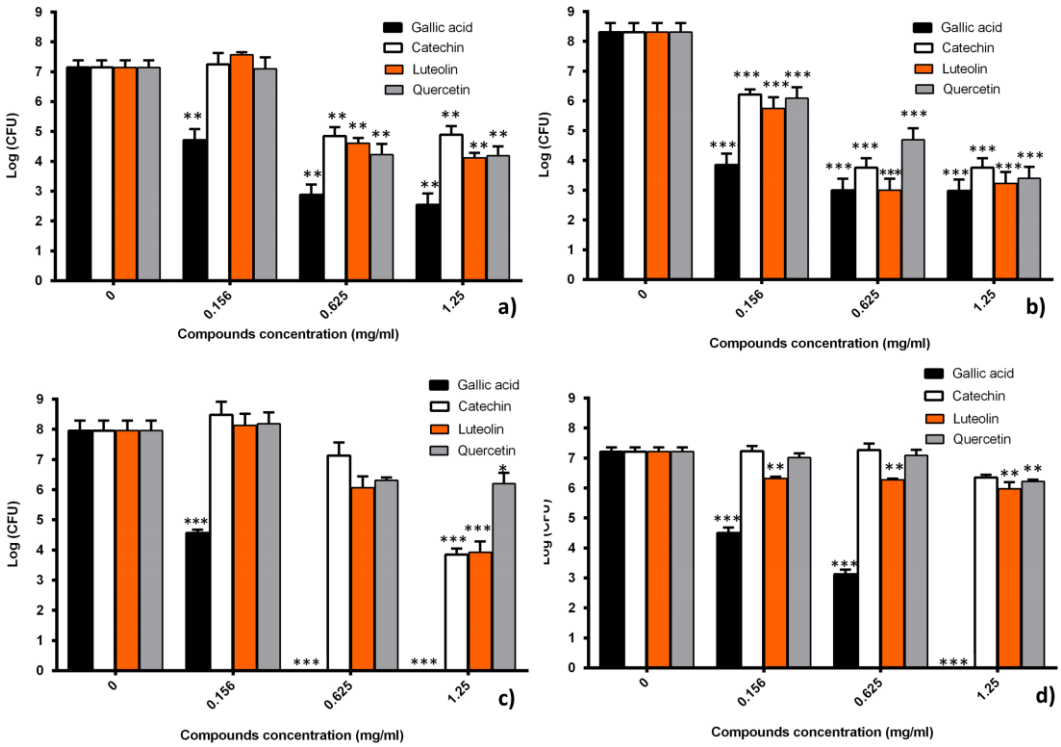

Figure 2.2 – Logarithmic number of *C. albicans* ATCC 90028 **(a)**, *C. glabrata* ATCC 2001 **(b)**, *C. parapsilosis* ATCC 22019 **(c)** and *C. tropicalis* ATCC 750 **(d)** grown in presence of increased concentrations of gallic acid, catechin, luteolin and quercetin, after 48 h. Error bars represent standard deviation. Concentrations that are significantly different (*P<0.05, **P<0.01, *** P<0.001) of each compound compared to control.

The cell culture results confirmed that gallic acid had the highest antifungal activity (*P<0.01* at all concentrations) against *Candida* planktonic cells (Figure 2.2 a-d). It should be noted that, gallic acid resulted in at least a 2 Log reduction in the CFUs of all tested species, at its lowest concentration (0.156 mg/ml). Interestingly, this phenolic acid also totally killed *C. parapsilosis* ATCC 22019 (Figure 2.2 c) and *C. tropicalis* ATCC 750 (Figure 2.2 d) planktonic cells at concentrations > 0.625 mg/ml (*P<0.001*) and 1.25 mg/ml (*P<0.001*), respectively. Despite, the fact that the mechanism of action of gallic acid on yeast cells has not been established, it can be proposed that it acts by disrupting the structure of the cell membrane and inhibiting the normal budding process [44–46].

Candida glabrata ATCC 2001 was the species that generally presented the highest initial reduction for all phenolic compounds tested with more than 2 Log reduction in CFUs at 0.156 mg/ml, (*P<0.001*) (Figure 2.2 b). However, in contrast to *C. tropicalis* ATCC 750 and *C. parapsilosis* ATCC 22019, gallic acid was unable to completely eradicate *C. glabrata* ATCC 2001 at any of the tested concentrations. Furthermore, this fungistatic effect was also observed against *C. albicans* ATCC 90028 (Figure 2.2 a). Catechin and luteolin had similar effects against *C. parapsilosis* ATCC 22019, causing > 3 Log CFU reduction at 1.25 mg/ml, (*P<0.001*), (Figure 2.2 c). In this study, *C. tropicalis* ATCC 750 was the species that showed the lowest susceptibility for all flavonoids, with less than 1 Log CFU reduction, even for the highest concentration tested (Figure 2.2 d).

Regarding all the results it was interesting to note that although gallic acid presented the highest antifungal activity, it was not present in *R. micrantha*, which was the plant extract that showed highest inhibition. Therefore, it should be highlighted that phenolic compounds have a synergistic effect within the extracts and so there was no direct relation in their activities.

2.3.3. Antifungal activity against biofilms of the phenolic compounds

In most natural environments, microorganisms exist predominantly as biofilms rather than as planktonic or free-floating cells [47]. So, the second aim of this work was to test the activity of the phenolic compounds against *Candida* species pre-formed biofilms. For that, the relative number of viable cells within the biofilm was evaluated by enumerating CFUs (Figure 2.3). Antimicrobial resistance by biofilms is a phenomenon consistently evident across model microbial systems [3,48] and is of great clinical relevance [49]. Hawser *et al*. [50], firstly demonstrated the resistance effect of *Candida* biofilms to traditional antifungal agents. As such, any evidence of activity against biofilm-associated organisms would represent an important new finding.

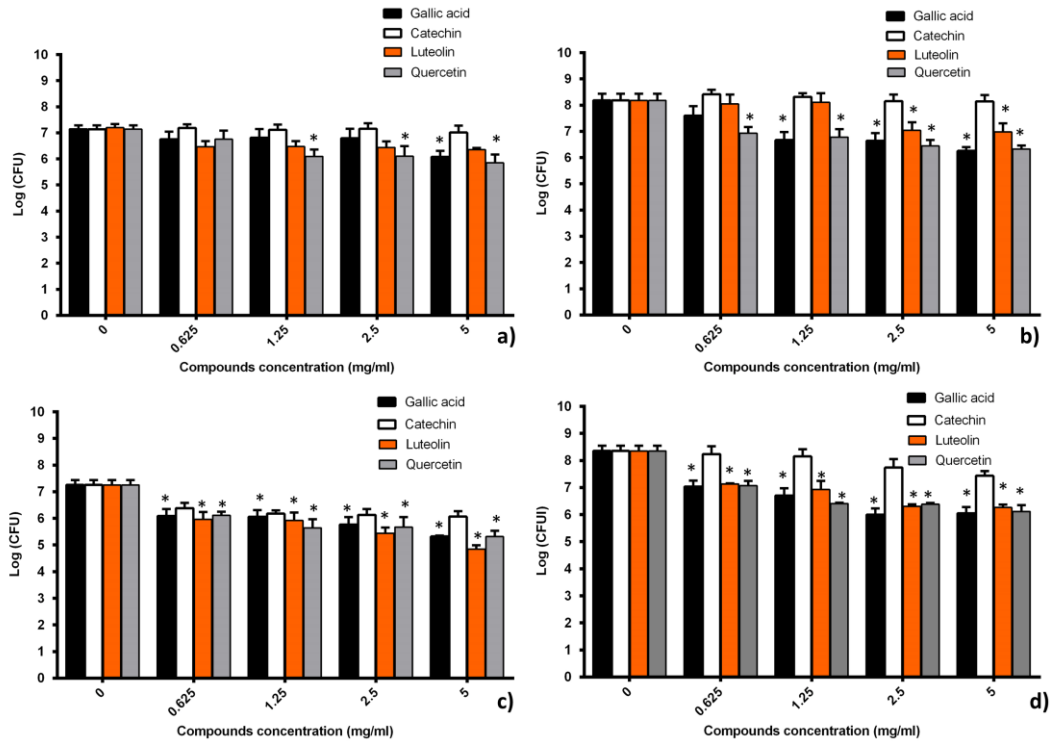

Figure 2.3 – Logarithmic number of *C. albicans* ATCC 90028 **(a)**, *C. glabrata* ATCC 2001 **(b)**, *C. parapsilosis* ATCC 22019 **(c)** and *C. tropicalis* ATCC 750 **(d)** cells in the biofilm treated with increasing concentrations of gallic acid, catechin, luteolin and quercetin, after 24 h biofilm formation in RPMI 1640. Error bars represent standard deviation. Concentrations that are significantly different (*P<0.05) of each compound control.

The effect of the phenolic compounds on *Candida* biofilms (Figure 2.3) revealed a decrease of susceptibility of these microorganisms comparatively to planktonic counterparts (Figure 2.2). Gallic acid, the phenolic compound that presented the highest effect for planktonic cells, luteolin and quercetin, were only able to reduce *C. glabrata* ATCC 2001 (Figure 2.3 b), *C. parapsilosis* ATCC 22019 (Figure 2.3 c) and *C. tropicalis* ATCC 750 (Figure 2.3 d) biofilms CFUs by 2 Log at the highest concentration tested (P<0.05).

In terms of species, *C. albicans* ATCC 90028 biofilms were the most resistant to all compounds, where the most active agents were gallic acid and quercetin (P<0.05) (Figure 2.3 a). Catechin were the phenolic compound which demonstrated the lowest effect, with only 1 Log CFU reduction at the highest concentration tested with *C.*

parapsilosis ATCC 22019 (Figure 2.3 c).

Biofilms are organised and structured communities embedded within a matrix of extracellular material [47]. The structure of the *Candida* biofilm matrix and its composition is known to be highly species dependent [47,51]. For example, *C. albicans* ATCC 90028 biofilm structure in polystyrene plates involves, generally, two distinct layers: a thin, basal yeast layer and a thicker, less compact hyphal layer and *C. parapsilosis* ATCC 22019 biofilms are thinner, less structured, and consist exclusively of aggregated blastospores [52], which could be the reason for the different results obtained for each *Candida* species.

2.4. Acknowledgments

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Chapter III

Candida albicans promotes invasion and colonisation of Candida glabrata in a reconstituted human vaginal epithelium

The work presented in this chapter is submitted for publication:

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Abstract

Candida species are the most common cause of mucosal and invasive fungal infections in humans. Vaginal candidosis (VC) is an infection of the vaginal epithelium caused primarily by *Candida albicans.* However, *Candida glabrata* has also emerged as an important pathogen in human infection. Relatively little is known about the role of *C. albicans* and *C. glabrata* during co-infection of vaginal epithelium, which is important, given potential synergistic effects and relative difference in antifungal susceptibilities. The aim of this study was therefore to investigate the *in vitro* co-infection of a reconstituted vaginal epithelium (RHVE) by *C. albicans* and *C. glabrata*.

The ability of both species to invade and colonise the (RHVE) was examined using species-specific peptide nucleic acid (PNA) probe hybridisation, confocal laser scanning microscopy (CLSM) and a novel qRT-PCR protocol for *Candida* quantification in the tissues. RHVE damage was evaluated by measuring lactate dehydrogenase (LDH) activity. *Candida* virulence gene expression (*HWP1*, *ALS*, *EPA*, *PLB*, *PLD* and *SAP*) was evaluated by quantitative RT-PCR. The results confirmed the effectiveness of RHVE as an *in vitro* model to study *Candida* virulence attributes and showed that whilst both species induced damage to the RHVE, this was notably less with *C. glabrata*. Interestingly, there was a significant increase in *C. glabrata* RHVE colonisation and invasiveness when it was added to the tissue with *C. albicans*. The extent of RHVE damage caused by the two species appeared to be primarily dependent on the process of invasion. Of the virulence genes assayed, *HWP1*, *PLD1* and *ALS3* were deemed to be most associated with pathogenicity in the model.

In conclusion, for the first time, we have demonstrated that the RHVE model coupled with specific tools of analysis, allows assessment of *Candida* colonisation and invasion in single and co-infection. Using this model we have demonstrated that *C. albicans* enhanced *C. glabrata* colonisation, invasion and tissue damages, which was also evidenced by the expression of virulence genes.

3.1. Introduction

Several *Candida* species can colonise human mucosal surfaces as harmless commensals. Normally, infection (candidosis) does not occur due to the host's effective innate and adaptive immune responses. However, debilitation of the host often results in candidosis, which most frequently manifests as superficial infections of moist mucosal surfaces, such as those of the vagina and oral cavity [1,2]. Amongst the many causes of vaginitis, vaginal candidosis (VC) is the second most common after bacterial vaginosis. VC is diagnosed in up to 40% of women with vaginal complaints in the primary care setting [3]. Approximately 75% of women experience at least one episode of VC during their lives [4,5].

Candida albicans is regarded as the most frequent species involved in both colonisation and infection [2,6]. However, there has been a notable increase in the relative proportion of infections caused by non-*Candida albicans Candida* (NCAC) species [6]. An important species in this regard is *Candida glabrata*, which is now the second leading cause of both blood stream and mucosal candidosis in the United State of America [6,7]. *C. glabrata* is also the primary NCAC species emerging in VC, accounting for up to 14% of infections in immune-competent women [8,9].

Several model systems have been used to study *Candida*-host interactions, including murine models [10]. Murine models of oral candidosis have identified several potential virulence factors that may play a role in pathogenesis [11,12]. However, since *C. albicans* does not naturally colonise mice, murine models do not necessarily adequately reflect human candidosis [13], which makes results difficult to interpret.

The human epithelium is a complex multi-layered, multi-cellular tissue consisting of increasingly differentiated epithelial cells [14,15] and in recent years, multi-layered organotypic three-dimensional *in vitro* culture systems have been developed to mimic human epithelium [16,17]. Reconstituted human vaginal epithelium (RHVE), is a biological product commercialised by SkinEthic Laboratories (Nice, France) and is based upon the A431 vaginal squamous cell carcinoma cell line. The epithelium has successfully been

used to study *in vitro* mechanisms of tissue degradation and virulence gene expression following infection with *Candida* species [18].

Putative *Candida* virulence factors include the ability to exhibit morphological transition, adhere to host tissues and develop as biofilms, and secrete hydrolytic enzymes. In the case of the latter, secreted aspartyl proteinases (*SAPs*) are considered highly important enzymes in candidosis, which are able to degrade many human proteins at lesional sites, facilitating invasion of *Candida* into the epithelium [19]. Other tissue degrading enzymes produced by *Candida* include phospholipases, which hydrolyse one or more ester linkages of glycerophospholipids [20]. Host cell recognition and colonisation by *Candida* is also facilitated by adhesins, such as Agglutinin-Like Sequence (*ALS*) proteins and Hyphal Wall Protein (*HWP*) [21].

The aim of this study was to characterise *C. glabrata* and *C. albicans* co-infection of human epithelium by using an RHVE model, coupled with confocal laser scanning microscopy (CLSM) and quantitative real-time polymerase chain reaction (qRT-PCR). Analysis of *HWP*, *ALS*, phospholipase B and D (*PLB* and *PLD*), *SAP* and epithelial adhesion (*EPA*) virulence gene expression was also undertaken.

3.2. Materials and methods

3.2.1. Microorganisms

Three clinical isolates of *Candida* were used in this study and isolated from the oral cavity (*C. albicans* 324LA/94 and *C. glabrata* D1) and vagina (*C. glabrata* 585626). The oral isolate of *C. albicans* was obtained from the culture collection of the School of Dentistry, Cardiff, UK and the oral strain of *C. glabrata* was acquired from the biofilm group of the Centre of Biological Engineering, Minho University (Braga, Portugal). The strain isolated from vaginal infection was gifted from the culture collection of the Hospital de Braga (Portugal). In addition to these clinical isolates, two reference strains, namely, *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 (obtained from the American Type Culture Collection) were also included in this study. The *Candida* strains were selected based on their previously determined ability to invade a reconstituted human oral epithelium [22]. The identity of all strains was confirmed by PCR-based sequencing using specific primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´)[23].

3.2.2. Culture of *Candida*

For each experiment, isolates were cultured on Sabouraud's Dextrose Agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. The isolates were then sub-cultured overnight in Yeast Nitrogen Base medium (YNB; BD Diagnostics, Cowley, UK) supplemented with 0.5% glucose (w/v) at 37°C under agitation at 120 rev/min. After incubation, cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice in 10 ml of Phosphate Buffered Saline (PBS; pH 7, 0.1 M). The cellular density of each strain was adjusted to $2x10^6$ cells/ml in SkinEthic maintenance medium (SkinEthic Laboratories, Lyon, France) using an improved Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany).

3.2.3. Infection of a reconstituted human vaginal epithelium

To study *in vitro* single and co-infection of vaginal epithelium by *C. albicans* and *C. glabrata*, a commercially available reconstituted human vaginal epithelium (RHVE) (SkinEthic Laboratories; Nice, France) was used as an *in vitro* model of vaginal candidosis. RHVE tissue inserts (0.5 cm^2) were placed in 12-well tissue culture plates. As a negative control, 1 ml of maintenance medium was added to a RHVE tissue preparation. To study single species infection, 5 RHVE tissues were inoculated with 1 ml of standardised suspensions of each *Candida* isolate prepared as previously described.

In dual species studies, *C. albicans* ATCC 90028 (500 µl; 2×10^6 cells/ml) or *C. albicans* 324LA/94 was combined with one of *C. glabrata* D1, ATCC 2001, or 585626 (500 µl; 2×10^6 cells/ml). Each dual species experiment was repeated in 3 independent experiments.

All infected tissues were incubated at 37°C in a 5% CO_2 environment in saturated humidity for 24 h. After incubation, the tissue was rinsed twice in 1 ml of PBS to remove non-adherent *Candida* cells, and the tissue was then bisected, with one half being used for CLSM analysis and the other for qRT-PCR.

3.2.4. Histological techniques

The RHVE tissue for CLSM analysis was fixed in 2% (v/v) formalin and stored at 4°C until histological processing. Tissues were then dehydrated, cleared, and infiltrated with paraffin wax embedding material. The formalin-fixed, paraffin-embedded (FFPE) tissues were stored at room temperature. The tissues were cut (20 µm sections) and placed on Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed, and processed through xylene, ethanol, and water before peptide nucleic acid probe hybridization.

3.2.5. Peptide nucleic acid fluorescent *in situ* hybridization

Peptide nucleic acid probe fluorescence *in situ* hybridisation (PNA FISH) was employed on tissue sections using the Light PNA FISH™ kit (AdvanDx Inc., Woburn MA, USA). These species-specific probes were utilised to study the invasion of the RHVE by *C. albicans* and *C. glabrata*. The Light PNA FISH™ kit had previously been developed and evaluated using multicolour labelled fluorescent PNA probes targeting specific 26S rRNA sequences of *C. albicans* and *C. glabrata*. The assay was performed in accordance with the guidelines of the manufacturer. Tissue sections on microscope slides were overlaid with 1 drop of the respective PNA probes. After 90 min of incubation, in the dark in a humidified chamber at 55°C, unbound probe was removed by washing the slides using a previously warmed wash solution at 55°C for 30 min. The preparation was then mounted with a medium suitable for fluorescence microscopy, which also contained 4',6-diamidino-2-phenylindole (DAPI) for detection of epithelial cell nuclei (Vectashield, Vector laboratories, California, USA). Samples were examined by CLSM, where *C. albicans* and *C. glabrata* were represented by bright green and red fluorescence, respectively.

3.2.6. Confocal laser scanning microscopy (CLSM)

Tissue sections (20 µm) hybridised with PNA probes were observed by CLSM, using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) to analyse the level of colonisation and morphological characteristics of *C. albicans* and *C. glabrata* in single and co-infection of the surface of RHVE tissues. Sections were scanned through the full depth using appropriate settings for single, double or triple channel fluorescence recordings. Fluorescein-labelled PNA was used for detection of *C. albicans* (laser excitation line 492_{nm} and emissions detected 520_{nm}); Tamara-labelled PNA was used for detection of *C. glabrata* (laser excitation line 565_{nm} and emissions detected 580_{nm}) and DAPI (laser excitation line 485_{nm} and emissions detected $410\text{-}485_{nm}$) for nuclear context of keratinocytes. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or maximum intensity type

constructions. A specific classification scale was used to express the level of *Candida* RHVE colonisation. Colonisation categories were as follows: extensive (fungal elements totally covered the surface); moderate (fungal elements covered a large proportion of surface) and sparse (fungal elements covered only limited areas of surface). Similarly, the following categories defined the level of *Candida* RHVE invasion: high (fungal elements totally invaded all RHVE keratinocytes layers); moderate (fungal elements moderately invaded the first RHVE top keratinocytes layers) and low (fungal elements only formed isolated clusters in the first RHVE keratinocytes layer).

3.2.7. Lactase dehydrogenase (LDH) activity

The extracellular activity of LDH released from RHVE cells was monitored as an indicator of tissue cell damage. LDH released in the maintenance medium of the control RHVE, devoid of *Candida*, as well as infected RVHE was measured at 24 h using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, Madison, WI, USA). LDH activity from *Candida* cells in a planktonic control (prepared as described for the infection model but devoid of RHVE) was subtracted from the LDH activity of the tissues infected with the different single and co-infection *Candida* species. The LDH released during single or co-infection with the different *C. albicans* and *C. glabrata* strains was then expressed as relative LDH activity to the untreated control tissue. LDH activity was analysed spectrophotometrically (FLUOstar OPTIMA, BMG Labtech, Ortenberg/Germany) by measuring the NADH disappearance rate at 544_{nm} excitation and 590_{nm} emission during the LDH-catalysed conversion of pyruvate to lactate. All experiments were performed in triplicate.

3.2.8. Quantification of *Candida* species in RHVE tissue sections

I- DNA extraction

After histological processing, four tissue sections of 20 µm were cut from each 12 FFPE tissues and placed in a sterile 1.5 ml microcentrifuge tubes (Eppendorf AG, Hamburg, Germany). Paraffin was removed using 1 ml of xylene and the tubes vigorously vortexed for 10 seconds. After centrifugation (13,000 *g* for 2 min), the supernatant was discarded and the residual xylene removed from the tissue pellet by adding 1 ml of absolute ethanol. After repeat centrifugation, the supernatant was carefully removed. The tubes were left open for 10 min at room temperature and DNA for PCR analysis was extracted from the tissue pellet using a commercial DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen).

II- Quantification of DNA by Real-time PCR

Candida were quantified using real-time PCR in an ABI Prism 7000 (Applied Biosystems, USA). For each sample, 5 µl of extracted DNA was added to a PCR solution containing 12.5 µl of 2× qPCR Master Mix and 0.25 µl of reference dye (SYBR® Green JumpStart™ *Taq* ReadyMix™, Sigma-Aldrich, St Louis, USA), 2 µl of 3 µM of each primer (Sigma-Aldrich, St Louis, USA) (Table 3.1) and 3.25 µl of dH_2O (Promega, Madison, WI, USA). Negative controls were performed using a reaction mixture with dH_2O (Promega, Madison, WI, USA) substituting for the template DNA. Template DNA for each positive control was obtained from FFPE tissues after the step of DNA extraction described above. PCR cycling conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 s primer annealing at 60°C for 30 s and extension at 72°C for 30 s. In each cycle, a dissociation stage at 60°C was run to generate a melting curve for verification of amplification product specificity.

Table 3.1 – Primers used for Real-time PCR screening for *C. albicans* and *C. glabrata*

Sequence (5'→ 3')	Primer	Target
GAGCGTCGTTTCTCCCTCAAACCGCTGG	Forward	*C. albicans* strains
GGTGGACGTTACCGCCGCAAGCAATGTT	Reverse	
ATTTGCATGCGCTTGCCCACGAATCC	Forward	*C. glabrata* strains
ACGTCTGATCCAATCAATGGCTGGTGA	Reverse	

Calibration curves (Ct vs. Log cells) for each *Candida* isolate were constructed using the same PCR protocol as described above (data not shown). For these, serial dilutions of the *Candida* were prepared and the DNA for PCR analysis extracted from the planktonic cell pellet using the DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen, Crawley, UK) with some modifications.

3.2.9. Analysis of candidal virulence gene expression

I- RNA extraction

For gene expression analysis, each *Candida* was cultured as previously described. For RNA extraction, fresh RHVE tissue removed with a scalpel directly from the plastic scaffold was placed in a 1.5 ml microcentrifuge tube (QIAshredder®, Qiagen, Crawley, UK) with 600 µl of RLT buffer containing 500 µl of glass beads (0.5 mm diameter) and 1/100 (v/v) of *β*-mercaptoethanol. This final mix was homogenised twice for 30 s using a Mini-Bead- Beater-8 (Stratech Scientific, Soham, UK) to lyse cells. The RNeasy Mini Kit (Qiagen, Crawley, UK) was then used to complete total RNA extraction from the tissue according to the manufacturer's recommended protocol. To remove any DNA contamination, samples were treated with RNase-Free DNase I (Invitrogen, Carlsbad, USA). The RNA extraction was performed on three different independents assays.

II- Synthesis of cDNA

From each sample, 0.5 µg of RNA was used for complementary DNA (cDNA) synthesis. RNA (14 µl) was incubated with 1 µl (50 µg/ml) of random primer mix (Promega, USA) in RNase free dH_2O at 70°C for 5 min. To this solution, 10 µl of a master mix containing 5 µl of M-MLV Reaction buffer (5×), 1 µl of each deoxynucleoside triphosphate (dNTP, 10 µM), 1 µl of RNasin Plus RNase inhibitor, 1 µl of M-MLV enzyme and 2 µl RNase free dH_2O was added. All reagents used in these experiments were from Promega Corporation, UK. Synthesis of cDNA was performed at 37°C for 1 h.

III- Primer design

Primers for 'housekeeping genes' (*ACT1*_alb and *ACT1*_gla), and putative virulence genes (*ALS*, *HWP, EPA, PLB, PLD* and *SAP)* were designed using Primer3 web software (http://fokker.wi.mit.edu). Full-length gene sequences were obtained from the *C. albicans* and *C. glabrata* database http://www.candidagenome.org [24]. The specificity of each primer was confirmed by comparing its respective sequences to the *C. albicans* and *C. glabrata* database using BLAST [25]. To verify the specificity of each primer pair for its corresponding target gene, PCR using the various primer pairs was applied to genomic DNA extracted from each of the *Candida* strains.

The sequences of the primers developed in the present study are provided in Table 3.2 and 3.3.

Table 3.2 – Forward (FW) and reverse (RV) housekeeping primers used for Real-time PCR

	Sequence (5'→ 3')	Orientation	Target
Housekeeping gene (*C. albicans*)	TGCTGAACGTATGCAAAAGG	FW	*ACT1*_alb
	TGAACAATGGATGGACCAGA	RV	
Housekeeping gene (*C. glabrata*)	TTGCCACACGCTATTTTGAG	FW	*ACT1*_gla
	ACCATCTGGCAATTCGTAGG	RV	

Table 3.3 – Forward (FW) and reverse (RV) primers used for Real-time PCR

	Sequence (5'→ 3')	Orientation	Target
***HWP* gene**	TCTACTGCTCCAGCCACTGA	FW	*HWP1*
	CCAGCAGGAATTGTTTCCAT	RV	
***ALS* genes**	CCCAACTTGGAATGCTGTTT	FW	*ALS1*
	TTTCAAAGCGTCGTTCACAG	RV	
	GCACTTCATTGACTGGAGCA	FW	*ALS2*
	TCATTGTTGCCACCTTGTGT	RV	
	CTGGACCACCAGGAAACACT	FW	*ALS3*
	GGTGGAGCGGTGACAGTAGT	RV	
	TCCACAGTTTCTCGTCCACA	FW	*ALS4*
	ATTGCCACGCTTGTTTTACC	RV	
	GTTCAGACATGCCATCATCG	FW	*ALS5*
	CCAAGTGATCAGGGTGGACT	RV	
	ATCGGAAGCTCCAAATTCCT	FW	*ALS6*
	AGGATGTTTAGTGGCGGATG	RV	
	GACCTTTTGTGGATGCGATT	FW	*ALS7*
	TTTTCTGGAGTCGGGAAATG	RV	
	GTGCCACAATGTGAGAATGG	FW	*ALS9*
	GTGCCACAATGTGAGAATGG	RV	
***EPA* genes**	ATGTGGCTCTGGGTTTTACG	FW	*EPA1*
	TGGTCCGTATGGGCTAGGTA	RV	
	TTATGCCGTATGGGGTTCTC	FW	*EPA6*
	GAGTCAACTGAGGCACACGA	RV	
	AGGATGCACACCCGAAATAG	FW	*EPA7*
	TTACCAGCCCCAAAATTCAC	RV	
Phospholipase B genes	GCTCTTTTCAACGAAGCGGTGT	FW	*PLB1*
	GCCATCTTCTCCACCGTCAACT	RV	
	CAATACTAGCCGCGTTGGGAAG	FW	*PLB2*
	GCCCATGAAAAACCCTGCATTA	RV	
	TCCCAATTGTTGTTGCTGATGG	FW	*PLB3*
	CCGCATTATCAAACCCACCAAT	RV	
	TCGTCCGGGTCTTCAAGTTCTC	FW	*PLB5*
	ATCTCCCGAATCCCCGTCTAAA	RV	
Phospholipase D gene	GCCAAGAGAGCAAGGGTTAGCA	FW	*PLD1*
	CGGATTCGTCATCCATTTCTCC	RV	
Secreted Aspartyl Protease genes	CAATGCTGCCACTGGACAAATC	FW	*SAP1*
	CAATTCAGCTTGGAAGGCATCA	RV	
	ACCGTTGGATTTGGTGGTGTTT	FW	*SAP2*
	ATTATTTGTCCCGTGGCAGCAT	RV	
	TGGTCCCCAAGGTGAAATCAAT	FW	*SAP3*
	TGGATCTTGTCCTTGACCAGCTT	RV	
	GTCAATGTCAACGCTGGTGTCC	FW	*SAP4*
	ATTCCGAAGCAGGAACGGAAAT	RV	
	ATCTTCCCGCACTTCCCAAAAT	FW	*SAP5*
	TCGCCGCTTTGAAAACCAATAC	RV	
	AAAATGGCGTGGTGACAGAGGT	FW	*SAP6*
	CGTTGGCTTGGAAACCAATACC	RV	
	ACGGGTGTTGTTTTGGATACCG	FW	*SAP9*
	GTCGACTGTTCTGCTGGAGTCG	RV	
	CCCGTTGATTCCAAAAGTCAGC	FW	*SAP10*
	TCGCCTATCGAAAACCCAAAGA	RV	

IV- Quantitative Real-time PCR

Real-time PCR was performed in 96-well plates (BrightWhite real-time PCR, Primer Design, UK) using the ABI PRISM 7000 (Applied Biosystems, USA). A total of 5 µl of 1:2 diluted cDNA samples and 20 µl of PCR solution (described above) were added to the plates with the respective virulence gene primers. Negative controls (dH_2O), as well as, non- reverse transcriptase controls (NRT) were included in each run. Real-time PCR was performed with an initial denaturation step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 15 s, primer annealing at 54°C for 30 s and extension at 72°C for 30 s. In each cycle, a dissociation stage at 60°C was run to generate a melting curve for verification of amplification product specificity. Control samples were included on each plate to ensure that multiple plates could be compared. The *ACT1* gene of *C. albicans* and *C. glabrata* had previously been used as a reference candidal housekeeping gene and were also used in this study.

All samples were run in triplicate. The Ct value of each sample was determined, and the relative gene expression levels calculated using the ΔCt method, which was normalized to the housekeeping genes described above.

3.2.10. Statistical analysis

Results were compared using one-way analysis of variance (ANOVA) by applying Tukey multiple-comparisons test, using Graphpad Prism program for Windows, version 6. All tests were performed with a confidence level of 95%.

3.3. Results

3.3.1. *In vitro* single and co-infection of RHVE

The surface colonisation and invasion of fresh RHVE by *Candida* was examined after 24 h incubation with *C. albicans* and *C. glabrata* strains in single and co-infection status (Figures 3.1, 3.2 and Table 3.4). The results showed that all strains were able to colonise the RHVE surface in single and co-infection. However, the level of colonisation was species, strain and combination dependent.

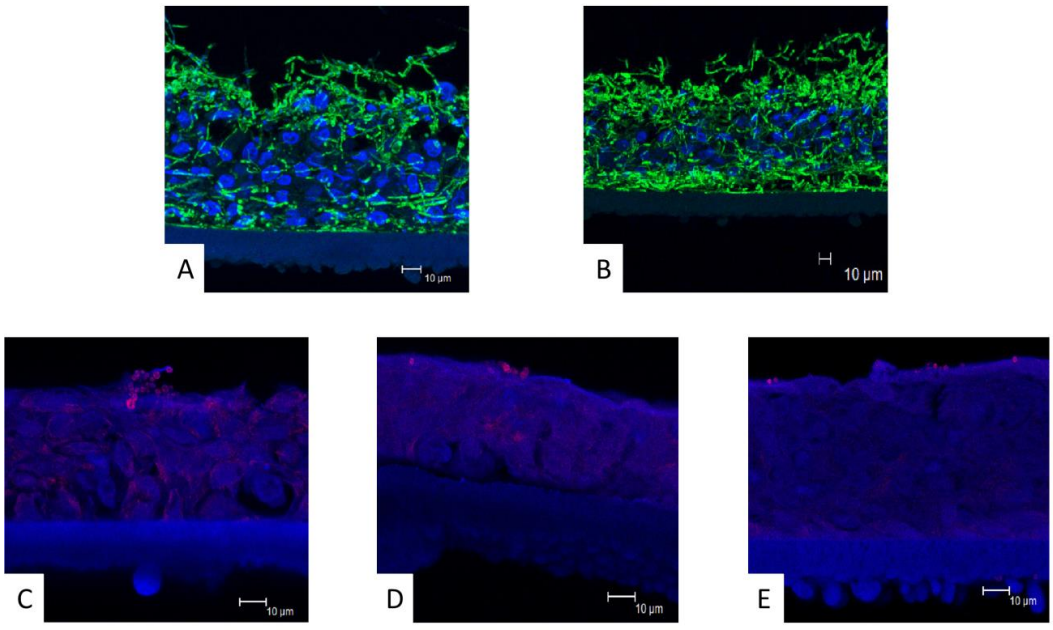


Figure 3.1 – *C. albicans* and *C. glabrata* single infection of RHVE after 24 h, assessed by CLSM and YTL PNA FISH™. **(A)** *C. albicans* ATCC 90028, **(B)** *C. albicans* 324LA/94 **(C)** *C. glabrata* ATCC 2001, **(D)** *C. glabrata* D1 and **(E)** *C. glabrata* 585626.

Candida albicans ATCC 90028 and *C. albicans* 324LA/94 (oral strain) exhibited the highest level of colonisation (extensive colonisation) of the vaginal epithelial surface in single and co-infection (Figures 3.1 A and Figure 3.2 A-C, Figures 3.1 B and Figure 3.2 D-F, respectively).

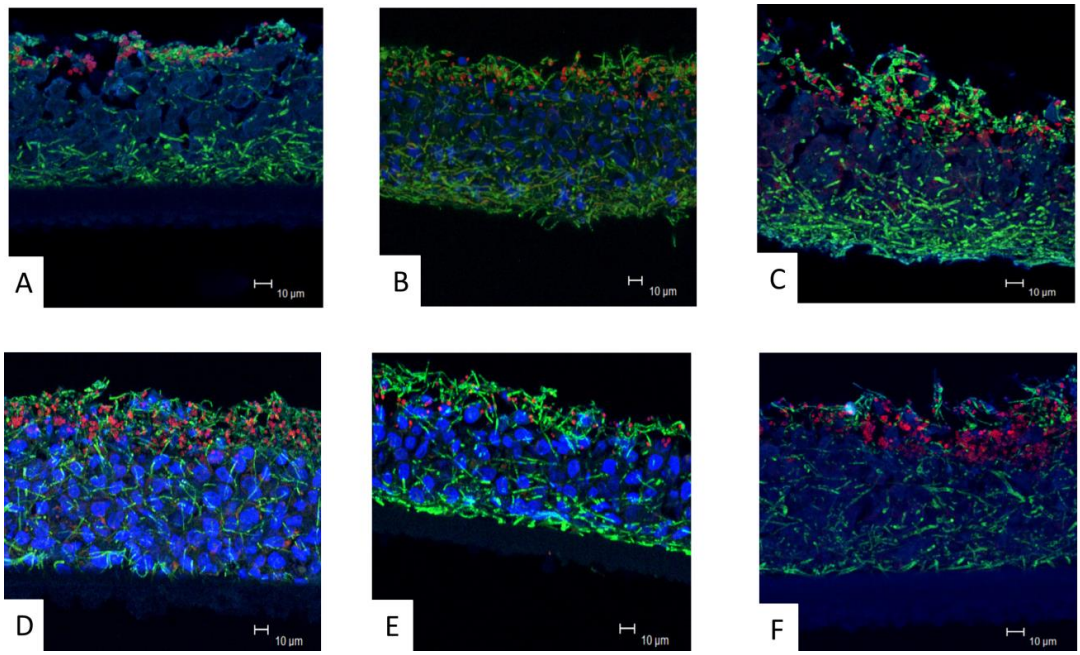


Figure 3.2 – *C. albicans* and *C. glabrata* strains co-infection of RHVE after 24 h, assessed by CLSM and YTL PNA FISH™. **(A)** *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001, **(B)** *C. albicans* ATCC 90028 and *C. glabrata* D1, **(C)** *C. albicans* ATCC 90028 and *C. glabrata* 585626, **(D)** *C. albicans* 324LA/94 and *C. glabrata* ATCC 2001, **(E)** *C. albicans* 324LA/94 and *C. glabrata* D1 and **(F)** *C. albicans* 324LA/94 and *C. glabrata* 585626.

Although all *C. glabrata* strains colonised the tissue in single infection, this was only sparse when compared with *C. albicans*. In general, after 24 h of single infection, only limited clusters of *C. glabrata* were detected on the surface of the keratinocyte layers (Figure 3.1 C, D, E). Additionally, in the presence of *C. albicans,* all *C. glabrata* (Figures 3.2) strains demonstrated increased RHVE colonisation. The co-infection of *C. glabrata* 585626 (vaginal strain) with *C. albicans* 324LA/94 (Figure 3.2 F) actually resulted in the *C. glabrata* having the same level of colonisation as encountered with *C. albicans* strains (*i.e.* extensive colonisation).

Furthermore, when tissue invasion was analysed, similar associations were evident. *Candida albicans* again had a high ability to invade the RHVE model, and it was possible to detect hyphal and yeast elements totally penetrating the RHVE layers (Figure 3.1 A-B). In single species infection involving *C. glabrata,* yeast invasion of the epithelium

was not apparent (Figure 3.1 C-E) and superficial detachment of keratinocyte layers was not evident. However, as with RHVE colonisation, when *C. glabrata* were combined with *C. albicans*, the level of invasion of *C. glabrata* was increased (moderate invasion in all combinations) (Figures 3.2).

Using real-time PCR, it was possible to quantify the number of *Candida* present in the RHVE sections (Table 3.4). *Candida albicans* was present in higher numbers (4-fold more) in the RHVE sections than *C. glabrata,* corroborating the CSLM observations. In both single and mixed species infections, *C. albicans* ATCC 90028 (4.76 ± 0.21 Log number of cells/ml) was present in significantly (*P<0.01*) higher numbers than *C. albicans* 324LA/94 (4.01 ± 0.24 Log number of cells/ml). No statistical differences were evident between the levels of *C. glabrata* in single species RHVE infection.

Compared with single species infection, it is important to note that the inoculum used for each individual species was halved in the co-infection studies (Table 3.4) and statistical comparison between single and co-infection assays was not possible. Despite this, it is important to emphasise that the number of *C. glabrata* cells increased considerably in all co-infection assays studied.

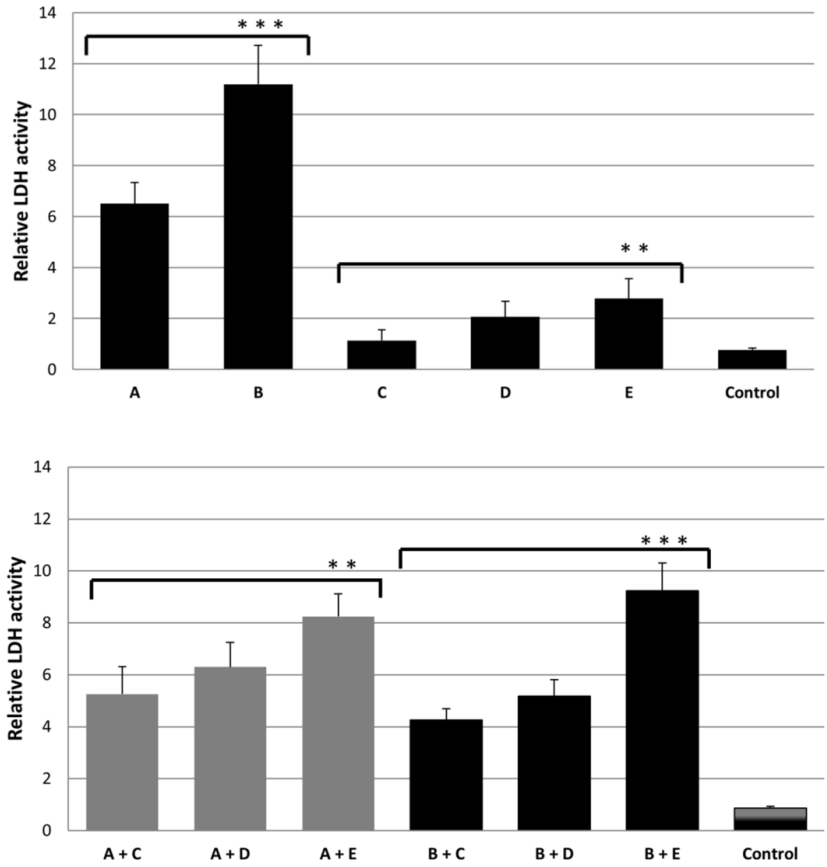
Table 3.4 – Summary findings of *C. glabrata* and *C. albicans* single and co-infection of reconstituted human vaginal epithelium at 24 h

Candida strain	Origin	Single Infection			Co-Infection *C. albicans* ATCC			Co-Infection *C. albicans* 324LA/94		
		Colonisation	Invasion	qRT-PCR (Log number of cells/ml)	Colonisation	Invasion	qRT-PCR (Log number of cells/ml)	Colonisation	Invasion	qRT-PCR (Log number of cells/ml)
							C. albicans ATCC			C. albicans 324LA/94
							C. glabrata strains			*C. glabrata* strains
***C. albicans* ATCC**	Reference	+++	High	4.76 ± 0.21	-	-	-	-	-	-
***C. albicans* 324LA/94**	Oral	+++	High	4.01 ± 0.24 **	-	-	-	-	-	-
***C. glabrata* ATCC**	Reference	+	Low	0.88 ± 0.41	++	Moderate	3.87 ± 0.39	++	Moderate	2.46 ± 0.35
							2.05 ± 0.23			1.97 ± 0.39
***C. glabrata* D1**	Oral	+	Low	0.89 ± 0.46	++	Moderate	3.99 ± 0.39	++	Moderate	3.01 ± 0.23
							1.89 ± 0.29			2.03 ± 0.22
***C. glabrata* 585626**	Vaginal	+	Low	1.30 ± 0.38	++	Moderate	4.66 ± 0.26	+++	Moderate	3.38 ± 0.26
							2.62 ± 0.21			2.55 ± 17

(+) sparse colonisation; (++) moderate colonisation; (+++) extensive colonisation;
** statistically different to reference strain (P<0.01).

3.3.2. LDH activity as an indicator of tissue damage

In order to determine the extent of RHVE damage induced by *Candida*, the levels of LDH activity were measured after 24 h of single and co-infection (Figure 3.3). In single infection, *C. albicans* caused the highest levels of tissue damage (*P<0.001*), with *C. albicans* 324LA/94 being significantly more damaging than the other strains (*P<0.001*). In the case of single species infection with *C. glabrata*, only *C. glabrata* 585626 (vaginal strain) resulted in a significant (*P<0.01*) increase in LDH activity compared to controls.



,*Strain/combination statistically different to reference strain (*P*<0.01; *P*<0.001);

Figure 3.3 – Relative lactate dehydrogenase (LDH) activity measured in the human vaginal epithelium tissue culture supernatant after 24 h incubation with different *C. albicans* and *C. glabrata* strains in single infection and co-infection (**A-** *C. albicans* ATCC 90028; **B-** *C. albicans* 324LA/94; **C-** *C. glabrata* ATCC 2001; **D-** *C. glabrata* D1; **E-** *C. glabrata* 585626).

In co-infection studies (Figure 3.3) it is again important to highlight that half of the number of each individual species was present in the starting inoculum. Interestingly, the

higher tissue damage previously seen with *C. albicans* 324LA/94 compared with *C. albicans* ATCC 90028 in single infection was not apparent in co-infection assays. *Candida albicans* ATCC 90028 and 324LA/94 combinations with *C. glabrata* 585626 resulted in significantly higher tissue damage ($P<0.01$; $P<0.001$, respectively), compared with other co-infection combinations. In all co-infection assays, the tissue damage demonstrated a similar profile with respect to that induced by the respective C*. glabrata* strains. The LDH values corroborated with the degree of tissue damage observed by CLSM (Figure 3.1, 3.2 and Table 3.4).

3.3.3. Gene expression

qRT-PCR revealed a wide range of expression of Hyphal Wall Protein (*HWP1*), Agglutinin-Like Sequence (*ALS*), Phospholipase B and D, Secreted Aspartyl Proteinase (*SAP)* (Table 3.5) and Epithelial Adhesin (*EPA*) genes (Table 3.6) for RHVE single and co-infection combinations. The results were expressed as the mean expression, relative to the expression of *ACT1* during RHVE infection. *ACT1* gene expression levels were constant in all assays.

HWP1 expression by *C. albicans* was up regulated during co-infection with all *C. glabrata* strains (Table 3.5). Expression of *ALS* genes was generally detected, however *ALS9* was not encountered in any of the analyses. Compared with single species infection, *C. albicans ALS1* expression was up-regulated for *C. albicans* ATCC 90028 in association with all *C. glabrata* strains. *ALS3* expression demonstrated similar results but for both *C. albicans* strains. Furthermore, for *C. albicans* 324LA/94 the other ALS genes were down-regulated when this strain was combined in infection with *C. glabrata*. Expression of Phospholipase B *and* D gene families by *C. albicans* was always detected in the samples (Table 3.5). All these genes were up-regulated during co-infection of *C. albicans* 324LA/94 and *C. glabrata* 585626. Expression of PLD1 was also up-regulated when *C. albicans* ATCC 90028 was co-infected with *C. glabrata* 585626. *SAP* genes were generally detected in all samples. However, *SAP3* was not expressed by *C. albicans* 324LA/94 during infection of RHVE. For this gene family, it is important to note that in co-infection, both *C.*

albicans with *C. glabrata* 585626 (vaginal strain) demonstrated a noticeable up -regulation of *SAP4-10* genes.

In contrast, Epithelial Adhesin gene expression by *C. glabrata* did not appear to correlate with the level of RHVE colonization and invasion (Table 3.6). In all mixed species combinations, expression of this gene family was shown to be principally down regulated, and in some cases absent.

Table 3.5 – Relative expression of *Hyphal Wall Protein 1* (*HWP1*), *Agglutinin-like Sequence* (*ALS*), *Phospholipase* (*B* and *D*) and *Secreted Aspartyl* Protease (*SAP*) gene families after 24 h of reconstituted human vaginal epithelium single and co-infection by different *Candida albicans* and *Candida glabrata* strains using quantitative RT-PCR

Gene	Single infection		Co-infection					
			C. albicans ATCC 90028			*C. albicans* 324LA/94		
	C. albicans ATCC 90028	*C. albicans* 324LA/94	C. *glabrata* ATCC 2001	C. *glabrata* D1	C. *glabrata* 585626	C. *glabrata* ATCC 2001	C. *glabrata* D1	C. *glabrata* 585626
HWP1	42 ± 7	50 ± 13	160 ± 13	220 ± 14	227 ± 27	521 ± 53	678 ± 46	750 ± 41
ALS1	216 ± 53	53 ± 7	859 ± 59	325 ± 22	426 ± 19	12 ± 5	18 ± 3	23 ± 2
ALS2	110 ± 11	128 ± 21	93 ± 5	141 ± 8	135 ± 9	67 ± 6	82 ± 10	75 ± 8
ALS3	345 ± 41	273 ± 29	575 ± 68	588 ± 24	1016 ± 166	308 ± 39	329 ± 16	485 ± 41
ALS4	224 ± 26	304 ± 33	201 ± 25	242 ± 14	187 ± 27	144 ± 13	187 ± 9	243 ± 8
ALS5	206 ± 21	208 ± 22	174 ± 8	129 ± 17	107 ± 9	106 ± 10	94 ± 7	65 ± 8
ALS6	374 ± 31	355 ± 40	484 ± 23	84 ± 5	222 ± 27	38 ± 4	44 ± 4	47 ± 5
ALS7	13 ± 2	32 ± 3	49 ± 7	80 ± 5	88 ± 3	23 ± 2	21 ± 3	ND
PLB1	31 ± 8	19 ± 5	38 ± 9	14 ± 3	34 ± 3	23 ± 2	10 ± 1	71 ± 10
PLB2	135 ± 10	102 ± 8	122 ± 18	99 ± 12	166 ± 18	76 ± 3	36 ± 2	206 ± 21
PLB3	218 ± 11	119 ± 3	179 ± 11	172 ± 9	238 ± 6	74 ± 3	18 ± 3	162 ± 20
PLB5	248 ± 6	159 ± 11	236 ± 8	126 ± 8	210 ± 2	94 ± 5	82 ± 3	366 ± 31
PLD1	278 ± 11	214 ± 4	270 ± 6	211 ± 12	390 ± 21	144 ± 5	94 ± 4	389 ± 30
SAP1	65 ± 9	21 ± 1	62 ± 8	39 ± 4	59 ± 2	26 ± 3	16 ± 1	33 ± 5
SAP2	182 ± 25	103 ± 6	196 ± 11	116 ± 6	134 ± 2	83 ± 4	34 ± 1	67 ± 6
SAP3	112 ± 11	ND	139 ± 15	78 ± 6	72 ± 3	ND	ND	ND
SAP4	118 ± 14	343 ± 10	224 ± 18	153 ± 14	266 ± 9	257 ± 10	134 ± 4	567 ± 24
SAP5	56 ± 6	123 ± 17	224 ± 15	214 ± 12	363 ± 22	187 ± 15	69 ± 7	298 ± 19
SAP6	63 ± 6	243 ± 10	104 ± 6	68 ± 4	117 ± 2	159 ± 12	115 ± 8	366 ± 25
SAP9	387 ± 17	329 ± 13	454 ± 24	351 ± 15	524 ± 4	244 ± 10	150 ± 9	555 ± 31
SAP10	103 ± 10	73 ± 3	114 ± 16	78 ± 6	180 ± 7	65 ± 6	31 ± 2	140 ± 14

ND, indicates that no gene expression was detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the respective *Candida* housekeeping gene product (ACT1).

Table 3.6 – Relative expression of Epithelial Adhesin (*EPA*) gene family after 24 h of reconstituted human vaginal epithelium single and co-infection by *Candida albicans* and *Candida glabrata* using quantitative RT-PCR

	Single infection			Co-infection					
				C. *glabrata* ATCC 2001		*C. glabrata* D1		*C. glabrata* 585626	
	C. glabrata ATCC 2001	*C. glabrata* D1	C. *glabrata* 585626	*C. albicans* ATCC 90028	*C. albicans* 324LA/ 94	*C. albicans* ATCC 90028	*C. albicans* 324LA/ 94	*C. albicans* ATCC 90028	*C. albicans* 324LA/ 94
EPA1	72 ± 5	110 ± 12	124 ± 10	60 ± 5	75 ± 3	70 ± 3	63 ± 5	96 ± 5	47 ± 7
EPA6	18 ± 1	25 ± 3	28 ± 5	13 ± 1	22 ± 2	ND	ND	ND	18 ± 3
EPA7	39 ± 1	49± 6	ND	18 ± 3	41 ± 8	22 ± 3	32 ± 2	ND	ND

ND, indicates that no gene expression was detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the respective *Candida* housekeeping gene product (ACT1).

3.4. Discussion

The mucosal epithelium is highly important in host defense and immune surveillance, as it is the first cell layer that initially encounters environmental microbes with pathogenic potential [26]. As a result, the characteristic feature of vaginal candidosis is chronic inflammation of the mucosa. In recent years, a number of studies have investigated colonisation and invasion of reconstituted human epithelium by *Candida* [18,19,27]. In this present study, the aim was to investigate colonisation and invasion of single and co-infection of *C. albicans* and *C. glabrata* in an *in vitro* reconstituted human vaginal epithelium (RHVE) model.

The work presented revealed that in single species infection (Figure 3.1 A, B and summarized in Table 3.4), both *C. albicans* strains tested extensively colonised and highly invaded the RHVE. Furthermore, *C. albicans* induced significant change in the vaginal keratinocytes structure, disruption of the superficial epithelium and cleft formation between the cells in the upper layers. These features have previously been reported in studies using RHOE [22,27–29]. After 24 h of incubation, CLSM revealed that *C. glabrata* were generally unable to invade the RHVE (very low invasion, Table 3.4) with few clusters of cells (Figure 3.1 C-E) within the first layers of the epithelium (sparse colonisation). These findings were in agreement with those of Schaller *et al*. 2002 [28] and Jayatilake *et al*. 2006 [30] who also classified *C. glabrata* as being non-invasive of RHOE. The differences between both species could be explained by the ability of *C. albicans* to form hyphae and/or pseudohyphae, and the absence of this property by *C. glabrata* [7,21]. Polymorphism and the movement of hyphae are indeed previously recognised factors enhancing RHOE colonisation and invasion [27,28,31].

Whilst the relative degree of *C. albicans* and *C. glabrata* RHVE colonisation and invasion was markedly species dependent, when the isolates were grouped according to clinical origin, there was no obvious association between origin and ability to colonise or invade RHVE. This is in agreement with other work performed with *Candida* species in an oral epithelium model [19].

In addition to CLSM observations, a molecular method was applied to enumerate the *Candida* able to colonise and invade RHVE. It was interesting to note that although the inoculum used in co-infection studies for each strain was halved compared with the single infection, the degree of colonisation and invasion of *C. albicans* in the presence of *C. glabrata* was not affected. Additionally, and as had previously been noticed using RHOE infection [22], colonisation by *C. glabrata* cells was highest when combined with *C. albicans*. Furthermore, *C. glabrata* invasion also increased in co-infections studies. Thus, the presence of *C. albicans* would appear to enhance invasiveness of *C. glabrata* in this model system, although the mechanism of this remains unclear. One possible explanation is that *C. glabrata* invasion is enhanced because of the damage induced to the epithelium by *C. albicans* hyphae (Figure 3.2). This would facilitate access of *C. glabrata* to lower epithelial layers. It is also possible that *C. glabrata* yeast could adhere to *C. albicans* hyphae and be transported to lower tissue layers. Kolenbrander *et al.*, 1993 [32] showed the ability of different oral microorganisms to adhere to each other (co-aggregation), which is known to be important in the development of dental plaque.

Adhesion of *C. albicans* to host cells is a key virulence mechanism and is associated with expression of *Hyphal Wall Protein* (*HWP*) [33]. It is known that the glutamine residues in the N-terminal domain of *HWP1* are cross-linked by host transglutaminase activity to unidentified host proteins and this results in covalent attachment of the yeast to host epithelial cells [34]. In this current research, qRT-PCR showed that in co-infection, both *C. albicans* strains exhibited up-regulation of *HWP1* compared with single infection. Based on the qRT-PCR findings it is tempting to speculate that RHVE invasion and colonisation by *C. glabrata* cells is enhanced by the presence of *C. albicans* hyphae following *HWP1* up-regulation.

Expression of adhesins may also be responsible for the different colonisation profile and therefore expression of genes of the Agglutinin-Like Sequence (*ALS*) and Epithelial Adhesins (*EPA*) family were also evaluated. Most ALS proteins have adhesin function [35], and the binding domain for most substrates is located in the N terminal. *ALS3* is a hypha-specific gene expressed by *C. albicans* hyphae and pseudohyphae, but not yeast [36,37]. The expression pattern of the *ALS* genes (Table 3.5) in *C. albicans*

RHVE single infection assays were generally similar to that observed in the co-infections, with exception of *ALS3*, which was highly increased. Since *ALS3* is *C. albicans* hyphal specific, its up-regulation in co-infection studies, might be reflected by increased hyphal production leading to enhanced invasion of *C. glabrata* yeast.

In vitro adherence of *C. glabrata* to epithelial cells is primarily mediated by *EPA* genes, which encode cell wall proteins of a large family of putative adhesins [38]. The *EPA* gene family consists of 17–23 members depending on the strain, and along with *EPA1*, at least two other genes have been shown to encode functional adhesins, *EPA6* and *EPA7* [39]. *EPA1* encodes a lectin that recognises host N-acetyl lactosamine containing glycoconjugates, aiding adhesion of yeast to mammalian surfaces. This gene exhibited highest expression in all the single *C. glabrata* assays presented here (Table 3.5). However, in co-infection, the majority of the *EPA* genes studied were down-regulated or absent, suggesting that these adhesins were not associated with increased RHVE colonisation or invasion by *C. glabrata* in the co-infection assays. Therefore, other factors might be responsible for this behaviour.

The degree of tissue damage caused by *Candida* during infection, was evaluated in single and co-infection (Figure 3.3) by LDH activity and results agreed with the CLSM observations (Figure 3.1, 3.2). Single species infection revealed that *C. albicans* generated more damage than *C. glabrata*. This result was expected given the fact that *C. albicans* is deemed more pathogenic [40]. LDH activity after co-infection yielded a profile similar to the *C. glabrata* in single infection, but understandably at higher levels because the presence of *C. albicans*. Furthermore, the previously observed differences between both *C. albicans* strains in single colonisation assays were not evident in co-colonisation. The differences between the *C. glabrata* strains were, however, still apparent with highest LDH activity occurring for *C. glabrata* 585626 combined with both *C. albicans*. In the case of *C. glabrata* 585626 and *C. albicans* ATCC 90028 co-infection, tissue damage was higher than with *C. albicans* ATCC 90028 alone, despite the initial inoculum of *C. albicans* ATCC 90028 being half that used in the single species infection. Based on these findings, it is evident that RHVE damage is species and strain dependent with potential synergism occurring in mixed species infection.

In order to better elucidate pathogenic differences, phospholipase and secreted aspartyl proteinase gene expression was measured. A variety of phospholipases (PL), are produced by *C. albicans* and the function of these enzymes includes the metabolism of phospholipids in biological membranes. Expression of these enzymes can therefore lead to disruption of membrane structure and function [41]. The current study showed that expression of *PLB and PLD* gene families was always detected, with *PLD1* expressed at a higher level in all single and *C. glabrata* 585626 co-infections assays, indicating a potential role of this factor in RHVE damage. However, these findings contrast with those of Malic *et al*. 2007 [27], where phospholipase gene expression did not correlate with RHOE invasion. Assumming that hyphal forms are more adept at epithelium invasion, expression of *PLD1* could be significant in this process as Hube *et al*., 2001 [42] reported that *PLD1* was necessary for the yeast-to-hypha transition in *Candida albicans*.

The most widely studied extracellular hydrolytic enzymes of *C. albicans* are *SAPs* [43–45]. This present study revealed that *SAP* gene expression by *C. albicans* was strain dependent in single and co-infection assays. From qRT-PCR analysis highly invasive *C. albicans* strains, in single infections were consistent producers of *SAP4–10*. Of note is that Naglik *et al*., 2003 [44] have shown that *SAP4–6* are important for yeast-to-hypha transformation. It is important to highlight that *SAP3* was not expressed by C*. albicans* 324LA/94, which was a 'high invader'. Korting *et al*., 2003 [46] determined that the epithelial cell damage by *C. albi*cans correlated with expression of *SAP3.* Based on the results of this current study and as indicated by Naglik *et al*., 2008 [10], it would appear that *SAPs* do not play a significant role in *C. albicans* invasion and damage of RHVE.

3.5. Acknowledgments

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Chapter IV

Effect of progesterone on Candida albicans vaginal pathogenicity

The work presented in this chapter is submitted for publication:

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Abstract

Despite Non-*Candida albicans Candida* species increasingly recognised as new pathogenic yeasts, it is generally still the case, that *Candida albicans* is the most virulent species. One of the most important candidal virulence factors is an ability to adhere to host surfaces and form biofilms. *Candida albicans* is responsible for the majority of cases of Vaginal Candidosis (VC), an infection which occurs mainly during the luteal phase of the menstrual cycle or during the pregnancy, when levels of progesterone are elevated.

The aim of this study was to determine the influence of progesterone on *C. albicans* virulence, namely biofilm formation and colonisation/invasion of a reconstituted human vaginal epithelium (RHVE). Biofilm formation on the RHVE was evaluated by enumeration of culturable cells, total mass quantification and scanning electron microscopy. The capacity of *C. albicans* strains to invade and colonise the tissue was examined by fluorescence microscopy using species-specific peptide nucleic acid (PNA) probe hybridisation, and quantitatively evaluated by RT-PCR *Candida* quantification methodology. Furthermore, gene (*BCR1* and *HWP1*) expression of biofilm and RHVE colonising cells was evaluated by quantitative RT-PCR. Results confirmed that progesterone reduced the capacity of *C. albicans* strains to form biofilms and to colonise and invade RHVE. Additionally, it was demonstrated that progesterone decreased expression of *BCR1* and *HWP1*, which are important virulence determinants of *C. albicans.* In conclusion, it was evident that progesterone can have a major influence on *C. albicans* pathogenicity on vaginal epithelial cells and may partly explain susceptibility of women to VC at different stages of the menstrual cycle.

4.1. Introduction

Yeast species of the genus *Candida* are responsible for 70-90% of human fungal infections, with *Candida albicans* accounting for approximately 50% of all yeasts isolated from clinical samples [1]. Interestingly, there has been a notable increase in the relative proportion of infections caused by non-*Candida albicans Candida* (NCAC) species [2]. However, *C. albicans* is still regarded to be the most virulent *Candida* species. Infection with *C. albicans* represents an important public health challenge with high economic and medical relevance due to the increased costs of care, time of hospitalisation and high levels of morbidity and mortality rates, especially in immunocompromised patients [3].

Vaginal candidosis (VC) is an opportunistic fungal infection caused by *Candida* species that affects between 29% and 45% of healthy women during their lifetime in European countries [4] and approximately 75% of women of gestation age in the United States [5,6].

The pathogenesis of VC involves the initial adherence of the yeast to the vaginal mucosa, followed by asymptomatic colonisation, ultimately leading to infection (symptomatic vaginitis) [7]. This occurs when the colonisation site of the host becomes favourable to the growth of yeast, which is normally restricted to asymptomatic colonisation and limited by immunological host responses. Conditions associated with enhanced colonisation include host immunosuppression, diabetes mellitus and pregnancy. The use of antibiotics, and oral contraceptives that contain high levels of oestrogen also seem to be contributory predisposing factors [5,8]. In the absence of these factors, clinical observations show that VC occurs predominantly during the luteal phase of the menstrual cycle, when levels of progesterone and oestrogen are elevated. In contrast, pre-menstrual girls and post-menopausal women who are not receiving hormone replacement therapy, rarely exhibit VC [9]. However, the mechanisms by which these hormones, act in VC are not fully known [10,11].

Amongst the putative virulence factors of *Candida* are the ability to express adhesins to adhere to host tissues and also produce biofilms [12]. Biofilms are microbial communities associated with biotic or abiotic solid surfaces. Cells in a biofilm display

phenotypes that are distinct from their free-living counterparts, including increased resistance to typical antifungal drugs [13,14]. Biofilm formation is, in part, dependent upon expression of the *C. albicans* transcription factor *BCR1* (biofilm cell wall regulator) [15]. Host cell recognition and colonisation by *Candida* is also facilitated by adhesins, such as Hyphal Wall Protein (*HWP*) [16], which is expressed by the hyphal form of *C. albicans*. Importantly, the effect of progesterone on expression of these virulence factors is not known and could be a contributory influence on *Candida* biofilm formation and vaginal human epithelial colonisation and invasion.

This current work aimed to investigate the influence of progesterone on *C. albicans* biofilm formation on a reconstituted human vaginal epithelium (RHVE). The colonisation/invasion of this tissue by *C. albicans* was assessed together with the expression of *BCR1* and *HWP1* during *C. albicans* biofilm development.

4.2. Materials and methods

4.2.1. Organisms and growth conditions

Two *Candida albicans* strains were used in this work, which were a reference strain *C. albicans* ATCC 90028 from the American Type Culture Collection (ATCC) and a vaginal isolate, namely *C. albicans* 558234, acquired from the biofilm group of the Centre of Biological Engineering, Minho University (Braga, Portugal) and previously isolated from a patient of the Hospital of S. Marcos, Braga, Portugal. The identity of the isolates were confirmed using CHROMagar®*Candida* (CHROMagar, France) and by PCR-based sequencing using specific primers ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) [17].

All *Candida* strains were subcultured on Sabouraud Dextrose Agar (SDA; Merck, Germany) at 37°C for 48 h. An inoculum of each strain, obtained from SDA plates, was suspended in 20 ml of Sabouraud dextrose broth (SDB; Merck, Germany) and incubated at 37°C for 18 h under agitation (120 rev/min). Then, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C and washed twice with 15 ml of phosphate buffered saline (PBS; pH 7, 0.1 M). Pellets were suspended in RPMI (Sigma, St Louis, MO), buffered to pH 4.0 and the cellular density adjusted for each experiment, using a Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany).

4.2.2. Effect of progesterone on *Candida albicans* biofilm formation

In order to study the effect of progesterone on *C. albicans* biofilm formation, progesterone (Sigma Saint Louis, Missouri, USA) was added at the beginning of the biofilm formation process. To the wells of 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium), 200 µl of *Candida* suspension containing $1x10^7$ cells/ml with 2 µM of progesterone in RPMI (pH 4) was added. The progesterone concentration was selected according to the levels present in the plasma of pregnant women in the third trimester [18]. The microtiter plates were incubated at 37°C under agitation (120 rev/min) for 24 h. Yeast cultures without progesterone and controls without *Candida* were also included. After incubation, the medium was aspirated and the biofilms washed once with 200 µl of PBS to

remove non-adherent cells. All experiments were performed in triplicate and in a minimum of three independent assays.

I- Quantification of cultivable cells from biofilms

Biofilms were physically removed from the wells using a pipette tip and the suspensions were vigorously vortex mixed for 2 min to disaggregate cells. Serial decimal dilutions of recovered cells in PBS were plated on SDA and incubated for 24 h at 37°C. The results were presented as total colony forming units (CFUs) per unit area (Log CFUs/cm^2) [19].

II- Quantification of biofilms biomass

Total biofilm biomass was quantified using a crystal violet (CV) staining methodology [19]. Biofilms in the microtiter plate wells were fixed with 200 µl of methanol for 15 min. The methanol was removed and the microtiter plates allowed to dry at room temperature. A 200 µl volume of crystal violet (CV; 1%, v/v) was then added to each well. After 5 min, excess CV was removed and the biofilms were gently washed (×2) with water. Finally 200 µl of acetic acid (33%, v/v) was added to each well to release and dissolve the CV stain. The absorbance of the CV solutions was then measured at 570_{nm} and results presented as absorbance per unit area (Abs/cm^2).

III- Characterisation of biofilm structure

The structure of biofilms and morphology of *Candida* in the presence and absence of progesterone was also characterised by scanning electron microscopy (SEM). Preparation of biofilms was as described above, but 24-well microtiter plates (orange Scientific, Braine-l´Alleud, Belgium) were used. Developed biofilms were dehydrated with increasing concentrations of ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and then air dried for 20 min. Samples were kept in a desiccator until analysis. Prior to observation, the base of the wells were removed and mounted on aluminium stubs, sputter coated with gold and imaged using an S-360 scanning electron microscope (Leo, Cambridge, USA).

4.2.3. Effect of progesterone on *Candida albicans* colonisation of RHVE

RHVE tissues (SkinEthic Laboratories, Lyon, France) were inoculated for 24 h with 1 ml of standardised *C. albicans* suspension (2×10^6 cells/ml) prepared in maintenance medium (SkinEthic Laboratories, Lyon, France) and adjusted to pH 4 in the presence and absence of 2 µM of progesterone. Tissues devoid of progesterone or *C. albicans* were also included. To determine the effects of progesterone on the measured parameters, the RHVE was also firstly incubated for 12 h with 1 ml (2×10^6 cells/ml) of each *C. albicans* strain in the absence of progesterone. The tissue was then washed (×1) with 1 ml of PBS to remove non-adherent cells and 1 ml of maintenance medium with 2 µM of progesterone was added to each tissue. All infected tissues were incubated at 37°C in a 5% CO_2 environment in a saturated humidity for the required time period. After incubation, tissues were washed (×2) with 1 ml of PBS to remove non-adherent *Candida*. Tissues were then bisected, with one half being used for fluorescence microscopy and the other for molecular studies.

I- Microscopic observation

The RHVE half of the tissue for microscopic analysis was fixed in 2% (v/v) formalin and stored at 4°C until histological processing. Tissues were then dehydrated, cleared, and infiltrated with paraffin wax embedding material. The formalin-fixed paraffin-embedded (FFPE) tissues were stored at room temperature. The tissues were cut (5 µm sections) and placed on Histobond+ coated microscope slides (Raymond A Lamb, East Sussex, UK), de-waxed, and processed through xylene, ethanol, and water before peptide nucleic acid probe hybridization (PNA FISH).

PNA FISH was employed on tissue sections using the Light PNA FISH™ kit (AdvanDx Inc., Woburn MA, USA). The species-specific PNA probes were used to ascertain the extent of invasion and colonisation of the RHVE by *C. albicans*. The assay was performed in accordance with the guidelines of the manufacturer. Tissue sections on microscope slides were overlaid with 1 drop of PNA probe. After 90 min of incubation in

the dark and in a humidified chamber at 55°C, unbound probe was removed by washing the slides using a previously warmed wash solution at 55°C for 30 min. The preparation was then mounted with a medium (*Vectashield, Vector laboratories, California, USA)* suitable for fluorescence microscopy, which also contained 4',6-diamidino-2-phenylindole (DAPI) for detection of epithelial cell nuclei.

Tissue sections (5 µm) hybridised with PNA probes were observed by fluorescence microscopy, using a BX51 Olympus fluorescence microscope coupled with a DP71 digital camera (Olympus Portugal SA, Porto, Portugal). A specific classification scale was used to express the level of *C. albicans* RHVE colonisation. Colonisation categories were as follows: extensive (fungal elements totally covered the surface); moderate (fungal elements covered a large proportion of surface) and sparse (fungal elements covered only limited areas of surface). Similarly, the following categories defined the level of *Candida* RHVE invasion: high (fungal elements totally invaded all RHVE keratinocytes layers); moderate (fungal elements moderately invaded the first RHVE top keratinocytes layers) and low (fungal elements only formed isolated clusters in the first RHVE keratinocyte layer).

II- Quantification of *Candida* cells in RHVE tissue sections

II.1- DNA extraction

From the remaining FFPE tissue, four tissue sections of 5 µm were cut and placed in sterile 1.5 ml microcentrifuge tubes (Eppendorf AG, Hamburg, Germany). Paraffin was removed using 1 ml of xylene and the tubes vigorously vortexed for 10 s. After centrifugation (13,000 *g* for 2 min), the supernatant was discarded and the residual xylene removed from the tissue pellet by adding 1 ml of absolute ethanol. After repeat centrifugation, the supernatant was carefully removed. The tubes were left open for 10 min at room temperature and DNA for PCR analysis was extracted from the tissue pellet using a commercial DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen).

II.2- Quantification of *Candida* by Real-time PCR

Candida albicans were quantified using real-time PCR employing a CF X96 Real-Time PCR System (Bio-Rad, Berkeley, USA). Each reaction mixture consisted of 10 µl of working concentration of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 0.2 µl of each primer (50 µM) designed previously (Forward-GAGCGTCGTTTCTCCCTCAAACCGCTGG and Reverse-GGTGGACGTTACCGCCGCAAGCAATGTT), and 4 µl of DNA, in a final reaction volume of 20 µl. Negative controls were performed using a reaction mixture with nuclease free H_2O (Cleaver Scientific Ltd, UK) substituting for the template DNA. Template DNA for each positive control was obtained from FFPE tissues after the step of DNA extraction described above. PCR cycling conditions consisted of an initial denaturation step at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 5 s and primer annealing at 60°C for 5 s. In each cycle, a dissociation stage of 60°C was run to generate a melting curve for confirmation the specificity of the amplification product. Calibration curves (Ct vs. Log cells) for each *C. albicans* strain were constructed using the same PCR protocol as described above. For these, serial dilutions of *Candida* were prepared using an improved Neubauer haemocytometer (Marienfild, Land-Könicshofen, Germany) and the DNA for PCR analysis extracted from the cell pellet using the DNA extraction kit (QIAamp® DNA FFPE Tissue, Qiagen, Crawley, UK) with some modifications.

4.2.4. Analysis of *HWP1* and *BCR1* gene expression in *C.* albicans biofilms

I- Candida RNA extraction

For gene expression analysis, biofilms were prepared in the presence (2 µM progesterone) and absence of progesterone as described above, in both microtiter plates and RHVE. In the case of microtiter plate biofilms, the culture medium was removed and the wells washed with 1 ml of PBS (pH 7) to remove non-adherent cells. The biofilms were then physically removed from wells by scraping with a pipette tip, and resuspended in 1 ml of PBS and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W to

separate the cells from the biofilm matrix. Cells were harvested by centrifugation at 8000 *g* for 5 min at 4°C and collected in a 1.5 ml microcentrifuge tube (QIAshredder®, Qiagen, Crawley, UK).

For RNA extraction from *Candida* adhered to tissues, fresh RHVE tissue was removed with a scalpel directly from the plastic scaffold and placed in a 1.5 ml microcentrifuge tube (QIAshredder®, Qiagen, Crawley, UK). To each microcentrifuge tube (QIAshredder®, Qiagen, Crawley, UK) 600 µl of RLT buffer containing 500 µl of glass beads (0.5 mm diameter) and 1/100 (v/v) of *ß*-mercaptoethanol was added. These mixes were homogenised twice for 30 s using a Mini-Bead-Beater-8 (Stratech Scientific, Soham, UK). After cell disruption, the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, USA) was used for total RNA extraction according to the manufacturer's recommended protocol. To remove any DNA contamination, samples were treated with RNase-Free DNase I (Invitrogen, Carlsbad, USA). The RNA extraction was performed on three different independents assays.

II- Synthesis of cDNA

To synthesise complementary DNA (cDNA), the iScript cDNA Synthesis Kit (Bio-Rad) was used according to the manufacturer's instructions. For each sample, 0.5 µg of the extracted RNA was used for cDNA synthesis. cDNA synthesis was performed firstly at 70°C for 5 min and then at 42°C for 1 h. The reaction was stopped by heating for 5 min at 95°C.

III- Gene selection and primer design for quantitative Real-time PCR

Primers for the 'housekeeping´ genes (*ACT1*_alb), and putative virulence genes (*HWP1, BCR1)* were designed using Primer3 web software (http://fokker.wi.mit.edu). Full-length gene sequences were obtained from the *C. albicans* database http://www.candidagenome.org [20]. The specificity of each primer was confirmed by comparing its respective sequences to the *C. albicans* database using BLAST [21]. To verify the specificity of each primer pair for its corresponding target gene, PCR using the various primer pairs was applied to genomic DNA extracted from each of the *C. albicans*

strains. The sequences of the primers developed in the present study are provided in Table 4.1.

Table 4.1 – Forward (FW) and reverse (RV) primers used for Real-time PCR

	Sequence (5'→ 3')	Orientation	Target
ACT1 gene (*C. albicans*)	TGCTGAACGTATGCAAAAGG	FW	*ACT1*_alb
	TGAACAATGGATGGACCAGA	RV	
***HWP1* Gene**	TCTACTGCTCCAGCCACTGA	FW	*HWP1*
	CCAGCAGGAATTGTTTCCAT	RV	
***BCR1* Gene**	CCAGCAGGAATTGTTTCCAT	FW	*BCR1*
	GGCTGTCCATGTTGTTGTTG	RV	

IV- Quantitative Real-time PCR (qRT-PCR)

qRT-PCR (CF X96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative levels of *HWP1* and *BCR1* mRNA transcripts in the biofilm samples, with *ACT1* used as a reference housekeeping gene. Each reaction mixture consisted of 10 µl (working concentration) of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 0.2 µl of forward and reverse primers (50 µM) (Table 4.1), 5.6 µl of dH_2O (Cleaver Scientific Ltd, UK), 4 µl of two-fold diluted cDNA samples together with the respective target gene primers. Negative controls (dH_2O), as well as, non- reverse transcriptase controls (NRT) were included in each run. Real-time PCR was performed with an initial denaturation step at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 5 s, and primer annealing at 58°C for 5 s. In each cycle, a dissociation stage at 60°C was run to generate a melting curve for verification of amplification product specificity. Control samples were included on each plate to ensure that multiple plates could be compared. The C_t value of each sample was determined, and the relative gene expression levels calculated using the ΔCt method, which was normalised to the housekeeping gene. Each reaction was performed in triplicate and mean values of relative expression were determined for each gene.

4.2.5. Statistical Analysis

Results were compared using a two-way ANOVA with the Bonferroni test, using GraphPad Prism 6 software. All tests were performed with a confidence level of 95%.

4.3. Results

4.3.1. Influence of progesterone on biofilm formation by *Candida albicans*

Initially, *Candida* growth curves were determined in RPMI (buffered to pH 4) with and without progesterone and no effect was observed in the normal yeast growth (data not shown). Then, biofilm formation of two different *C. albicans* strains, in the presence (2 μM) or absence of progesterone, was evaluated using CFU enumeration (Figure 4.1 A) and CV staining (Figure 4.1 B).

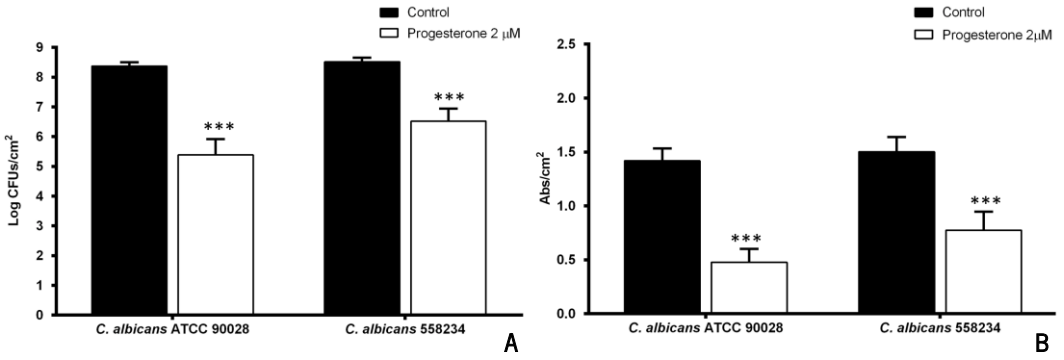

Figure 4.1 – Cell viability [Log (CFU/cm²)] **(A)** and absorbance values of **(B)** crystal violet solutions (Abs CV/cm²) obtained from 24 h biofilm of *C. albicans* strains formed in RPMI buffered to pH 4 in absence or presence of 2 μM of progesterone. Error bars represent standard deviation. *** Statistical difference obtained when compared with absence of 2 μM of progesterone (control) ($p<0.001$).

It was evident that the presence of progesterone significantly reduced the biofilm formation of both strains (Figure 4.1). The number of viable cells [Log (CFUs/cm²)] (Figure 4.1 A) decreased by approximately 3 Log in case of *C. albicans* ATCC 90028 ($p<0.001$) and 2 Log in the case of *C. albicans* 558234 ($p<0.001$). Similarly, a significant decrease in total biomass values ($p<0.001$) occurred in the presence of progesterone in RPMI medium for both *Candida* strains (Figure 4.1 B).

SEM analysis was used to examine biofilm structure and to determine possible differences concerning *Candida* morphological characteristics (Figure 4.2).

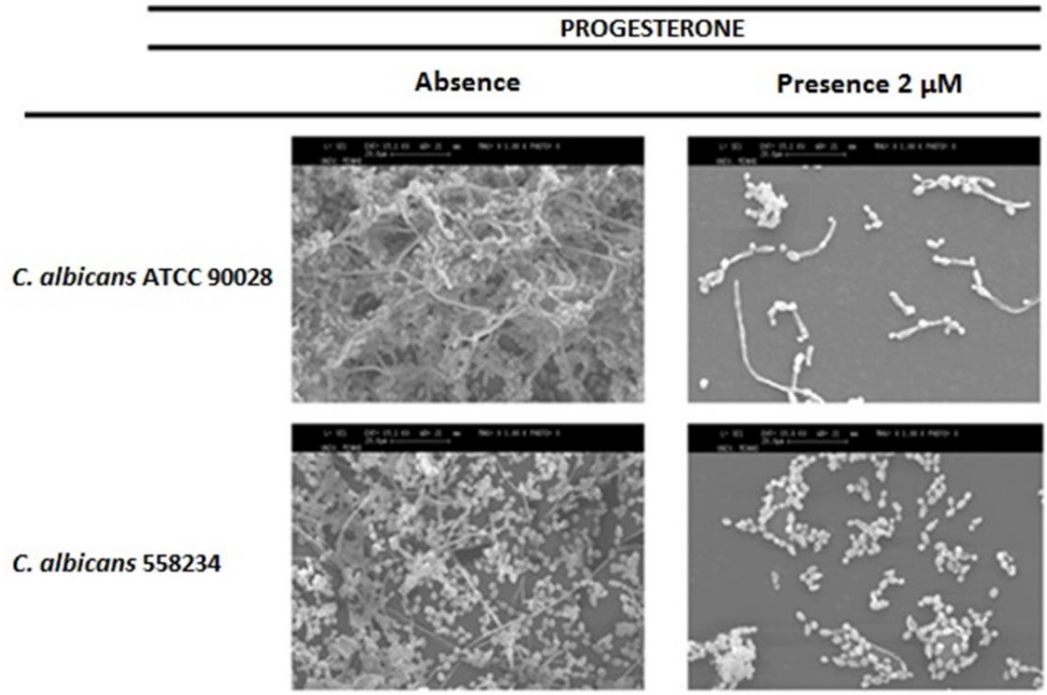

Figure 4.2 – Scanning electron microscopy of *C. albicans* 24 h biofilms formed in RPMI in the absence or presence of 2 µM of progesterone. Original magnification was ×1000.

Biofilms of *C. albicans* exhibited different structures and cell morphologies depending on culture environment conditions (Figure 4.2). Biofilms formed by both *C. albicans* strains in the absence of progesterone exhibited both yeast and hyphal morphology. However, biofilms of *C. albicans* ATCC 90028 formed a multilayered and compact biofilm that covered the entire surface. Biofilms of *C. albicans* 558234 consisted of non-contiguous cell aggregates with less hyphal forms. However, in the presence of 2 µM of progesterone, biofilms were greatly reduced in cell number and presence of hyphal forms.

Expression of the biofilm cell wall regulator 1 (*BCR1*) and hyphal wall protein (*HWP1*) genes were determined in an effort to understand the basis behind the observed biofilm changes following SEM. Results of these studies are presented in Figure 4.3 and expressed as the mean percentage expression, relative to the expression of *ACT1* during biofilm formation. *ACT1* gene expression levels were constant in all assays.

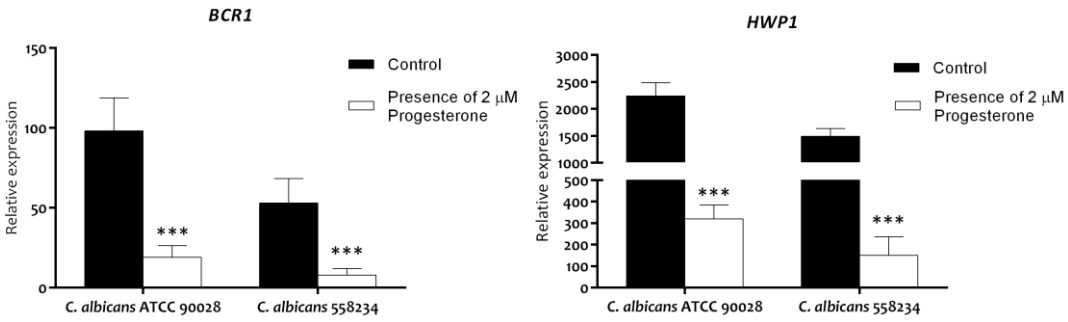

Figure 4.3 – Relative *BCR1* **(A)** and *HWP1* **(B)** expression obtained from 24 h biofilms of *C. albicans* formed in RPMI (pH 4), in the presence and absence of 2 μM of progesterone. Error bars represent standard deviation. *** Statistical difference obtained when compared with absence of progesterone ($p<0.001$).

The results (Figure 4.3) showed that both *BCR1* and *HWP1* expression varied in a strain dependent manner and with the presence of progesterone. It is important to highlight that in the presence of progesterone there was a statistically significant ($p<0.001$) down regulation of genes expression and for both strains.

4.3.2. Influence of progesterone in RHVE colonisation and invasion by *Candida albicans*

Candida albicans colonisation and invasion of RHVE was examined after 24 h of infection. Progesterone (2 μM) was added at two different time points, at the beginning and after 12 h of colonisation (Figure 4.4 and Table 4.2). The addition of progesterone at 12 h was performed to compare its influence in the development of RHVE infection by *C. albicans*.

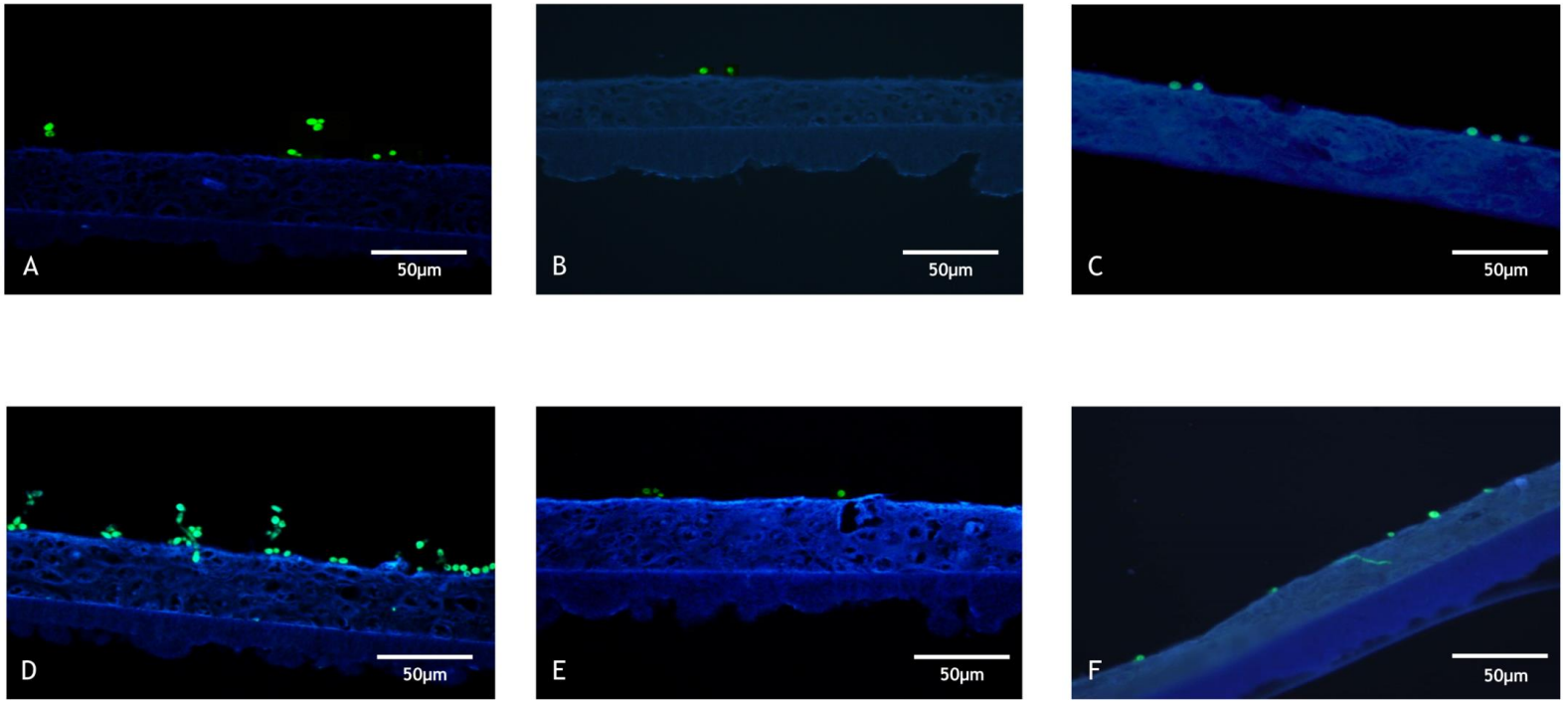

Figure 4.4 – *Candida albicans* RHVE infection after 24 h, assessed by fluorescence microscopy and YTL PNA FISH™. **(A)** *C. albicans* ATCC 90028 and **(D)** *C. albicans* 558234 adhered without progesterone, **(B)** *C. albicans* ATCC 90028 and **(E)** *C. albicans* 558234 adhered for 24 h in the presence of 2 µM of progesterone and **(C)** *C. albicans* ATCC 90028 and **(F)** *C. albicans* 558234 with addition of progesterone after 12 h of infection.

The results showed that for both strains and in all conditions tested, colonisation of the RHVE occurred. However, the degree of colonisation varied with strain and the presence of progesterone. *Candida albicans* 558234 (vaginal isolate) exhibited the highest level of colonisation (moderate) in the absence of progesterone (Figure 4.4 D). Under the same environmental conditions, *C. albicans* ATCC 90028 exhibited sparse colonisation (Figure 4.4 A). Additionally, both *C. albicans* strains demonstrated sparse colonisation in the presence of progesterone (Figure 4.4 B, E). In general, after 24 h and 12 h exposure to progesterone, only a few clusters of *C. albicans* were detected on the surface of the RHVE layers (Figure 4.4 C, F). Furthermore, only *C. albicans* 558234 appeared able to invade the epithelium (Figure 4.4 D, F).

Real-time PCR was used to quantify the number of infecting *C. albicans* under the different conditions tested (Table 4.2).

Table 4.2 – qRT-PCR quantification of *C. albicans* infecting the reconstituted human vaginal epithelium for 24 h in the presence or absence of 2 μM of progesterone

Progesterone [2 μM]	qRT-PCR (Log number of cells/ml)	
	C. albicans ATCC 90028	*C. albicans* 558234
Control	2.76 ± 0.11	3.56 ± 0.13
Addition at 0 h	2.17 ± 0.15 ***	2.77 ± 0.21 ***
Addition at 12 h	2.29 ± 0.17 ***	2.95 ± 0.13 ***

*** statistically different to absence of progesterone (P<0.001).

qRT-PCR showed that both *C. albicans* strains were present at higher numbers in the RHVE sections infected without progesterone (p<0.001), thereby supporting the findings of the fluorescence microscopy. There were no discernible differences in terms of *Candida* quantification when progesterone was added at time point 0 or at 12 h. It was

noted that *C. albicans* 558234 (vaginal strain) was always present in higher numbers than *C. albicans* ATCC 90028.

HWP1 expression was measured to better understand the effect of progesterone on RHVE colonisation and invasion. Figure 4.5 presents the mean n-fold expression levels of *HWP1* by *C. albicans* ATCC 90028 and *C. albicans* 558234 infecting the RHVE. The controls represent gene expression in absence of progesterone.

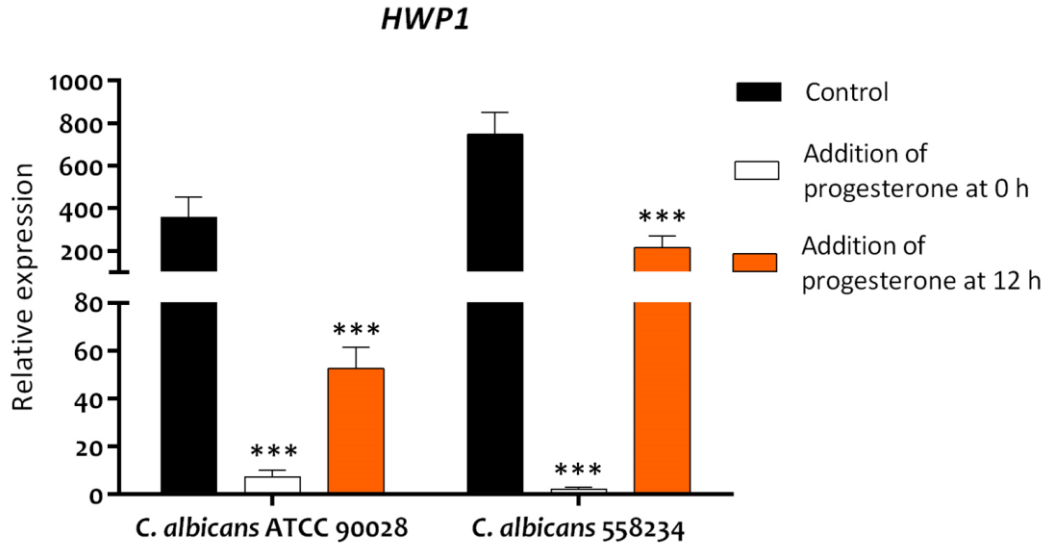

Figure 4.5 – Relative expression of the Hyphal Wall Protein 1 (*HWP1*) gene in the absence or presence (added at 0 h, or after 12h) of progesterone during *C. albicans* colonisation of reconstituted human vaginal epithelium. Error bars represent standard deviation. *** Statistical difference obtained compared to the absence of progesterone ($p<0.001$).

The results presented in Figure 5, indicate that *HWP1* expression was strain dependent and was significantly reduced by the presence of progesterone ($p<0.001$). *HWP1* expression was dependent on time of contact with progesterone, since after a pre-period of infection (12 h) without progesterone had higher expression of *HWP1* gene compared to addition at 0 h.

4.4. Discussion

Vaginal candidosis (VC) is an infection characterised by vaginal inflammation and the presence of *Candida* species. VC is the second most common cause of vaginitis, after bacterial vaginosis, and represents approximately one-third of all vaginitis cases [22]. The frequency of vaginal colonisation by *Candida* increases with predisposing risk factors such as pregnancy, antibiotic use, immunosuppression, diabetes and HIV infection [6,23].

Progesterone is a C-21 steroid hormone involved in the female menstrual cycle, pregnancy and embryogenesis. Progesterone levels are relatively low during the pre-ovulatory phase of the menstrual cycle, rise after ovulation, and are elevated during the luteal phase [24]. It has also been shown that *Candida* has oestrogen and progesterone receptors, that when stimulated, increase fungal proliferation [25]. Furthermore, women who take oral contraceptive pills (corresponding to constant high levels of progesterone and oestrogen) have a higher incidence of VCs [6].

This current study examined the influence of progesterone on *C. albicans* virulence factors, namely biofilm formation and colonisation/invasion of a reconstituted human vaginal epithelium (RHVE).

Initially, experiments were performed to examine the effect of both oestrogen and progesterone on *C. albicans* biofilm formation. However, preliminary studies with oestrogen failed to demonstrate any effects on biofilm formation and therefore the focus was directed towards progesterone.

The effect of progesterone was measured in terms of both number of viable cells and total biomass (Figure 4.1) and results were corroborated by SEM (Figure 4.2). Oestrogen has previously been shown to be the principle reproductive hormone that supports and sustains experimental vaginal *C. albicans* infection [10]. In contrast, progesterone was found to have no demonstrable effect on vaginal infection or on associated systemic and/or local immune responsiveness. However, in this present study a possible role for progesterone in VC has been highlighted. It is known that one of the central regulators of biofilm formation is the factor *BCR1* [26–28]. In these current studies expression of *BCR1* by *C. albicans* biofilms in the presence of progesterone was

significantly reduced (Figure 4.3 A). Furthermore, SEM (Figure 4.2) revealed a significant decrease in hyphal forms of *C. albicans*, in the presence of the hormone. In efforts to explain these findings, expression of hyphal wall protein (*HWP1*) was also measured and again, was found to decrease in the presence of progesterone (Figure 4.3 B). Hwp1 is a determinant of *C. albicans* pathogenicity, and involved in biofilm formation [29,30]. Additional studies have shown that reduced *HWP1* gene expression results in thin biofilms and a significant reduction in hyphal mass [31]. Based on the work of this present study, it is possible that progesterone affects *C. albicans* biofilm structure through modulation of the biofilm-associated genes, *BCR1* and *HWP1*.

In order to investigate the influence of progesterone on *C. albicans* vaginal pathogenicity, a commercially available reconstituted human vaginal epithelium (RHVE, Nice, France) was used. It was evident that both tested *C. albicans* strains could colonise the RHVE (Figure 4.4 and Table 4.2), with limited clusters of cells present within the upper layers of the epithelium and few filamentous forms. However, notably both colonisation and invasion substantially decreased in the presence of progesterone, without any apparent alteration of the RHVE (Figure 4.4 and Table 4.2). This results are in accordance with those of Špaček et al., 2007 [24] who showed that progesterone levels in luteal phase of recurrent VC patients (6.40 nmol/L) was lower compared with non-infected controls (34.2nmol/L).

Down-regulation of *HWP1* was evident in the presence of progesterone, as verified for biofilms, and was associated with reduced colonisation and invasion. The effect of progesterone on *HWP1* expression decreased slightly when added after 12 h of the infection process. Furthermore, colonisation and invasion of the RHVE by *C. albicans* increased slightly when progesterone was added 12 h post infection compared when added at start of RHVE infection. In 1992, Reed [25] had shown that *Candida* had receptors to progesterone and as these were activated, the degree of pathogenicity increased. The findings of this present study would appear to contradict the findings of Reed (1992) [25], as it would have been expected that a longer exposure to progesterone would have led to increased activation of the receptors leading to enhanced pathogenicity, this was not the case. Furthermore, *C. albicans* 558234 expressed higher levels of *HWP1* (Figure 4.5) when progesterone was added 12 h post infection, compared to those

infections with progesterone presence for 24 h. These findings were supported by hyphal detection using fluorescence microscopy (Figure 4.4 F).

In summary, progesterone was able to modulate *C. albicans* biofilm formation (cell viability, total biomass and structure), as well as the colonisation and invasion of RHVE. These effects may relate to the down regulation of the biofilm related genes *BCR1* and *HWP1* by progesterone. Additionally, *HWP1* gene expression by *C. albicans* infecting RHVE suggests an important role of progesterone in hyphal development. If these effects can be extrapolated to the *in vivo* situation then relative progesterone levels may play important roles in the progression of *C. albicans* vaginal infection.

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Chapter V

General conclusions and future work

5.1. General conclusions and future work

Treatment of candidosis is difficult which is partly due to the eukaryotic nature of fungal cells limiting the number of antifungals that are without host toxicity. Thus, amongst the traditional antifungal agents (azoles, polyenes or echinocandins) there are relatively few that are effective for clinical use. Furthermore, due to increased antifungal administration, antifungal resistance is becoming increasingly apparent. In order to overcome such problems, there is much interest in the potential use of natural extracts and compounds as alternative antifungal agents. In this current research, the phenolic extracts of *Castanae sativa, Filipendula ulmaria, Rosa micrantha* and *Cistus ladanifer,* exhibited promising antifungal activity against *Candida* species, suggesting that these compounds could potentially have a role in protection against fungal infections. Furthermore, it was demonstrated that these phenolic compounds, and particularly gallic acid, affected the growth of different planktonic *Candida* species. Catechin showed similar effect against *Candida albicans* ATCC 90028 at higher test concentrations. Gallic acid and quercetin demonstrated only slight activity against *Candida* species biofilms. Therefore, it should be highlighted that wild plants from the North Eastern of Portugal, and their compounds, represent important resources that could be utilised for the treatment of *Candida* that exhibit resistance to the common antifungal agents.

Future studies should continue to investigate these novel anti-*Candida* compounds derived from North Eastern Portuguese flowers and increase our understanding of their antifungal properties. Particular focus should be given to compounds that are effective against *Candida* biofilms, which are particularly problematic in clinical settings. The potential cytotoxicity of the most promising compounds should be assessed, in order to facilitate use as antifungals in human therapy.

Infections caused by *Candida* have increased in recent years, primarily due to the increasing number of immunocompromised patients, patients with diabetes mellitus and HIV associated diseases. *Candida* species are the second more common cause, after bacteria, of vaginal inflammation. Vaginal candidosis (VC) is an infection of the vaginal

mucous membranes caused most frequently by *C. albicans*. However, it is increasingly evident that non-*Candida albicans Candida* species are emerging as 'new pathogens' often in association with *C. albicans*. In this present study, it was confirmed that *C. albicans* was a much more extensive coloniser and invader of a reconstituted human vaginal epithelium (RHVE), compared with *C. glabrata*. However, it is important to highlight that co-infection of *C. albicans* with *C. glabrata* enhanced colonisation and invasiveness of the latter. To the candidate's knowledge, this represent the first report of *HWP1*, *ALS*, *EPA*, *PLB*, *PLD* and *SAP* gene expression in *C. albicans* and *C. glabrata* co-infection of vaginal epithelium and the results suggested an important role of *HWP1*, *PLD1* and *ALS3* in *C. albicans* and *C. glabrata* pathogenicity.

Although the present work provided some insights into *Candida* species vaginal tissue co-infection and associated virulence, some questions remain. It would be of value to investigate the host immune response to *C. albicans* and *C. glabrata* single and co-infection, with inclusion of macrophages or human peripheral blood lymphocytes within the model. Furthermore, the assessment of further co-infection models, using for example, some important vaginal pathogenic bacteria together with *Candida* species, would be interesting to enhance further the knowledge of the clinical condition, since VCs are often polymicrobial.

Another important factor in *Candida* pathogenicity is their ability to respond rapidly to environmental changes and this flexibility may allow these organisms to take advantage of impaired immunity in debilitated patients and facilitate disease. Consequently, the effect of an environmental factor (level of hormone) on the infection process was also evaluated. It was demonstrated that progesterone reduced *C. albicans* biofilm formation as measured by cell viability, total biomass and structure. Additionally, it was shown that the respective biofilm and hyphal related genes, *BCR1* and *HWP1,* were involved in biofilm modulation by progesterone. It was further shown that *C. albicans* was a higher RHVE coloniser in the absence of progesterone. *HWP1* gene expression by *C. albicans* infecting the vaginal epithelium would suggest an important role of progesterone in modulating the *HWP1* virulence factor in *C. albicans* pathogenicity. It would also be of benefit to enhance these

investigations using specific deletion mutants to increase the understanding of the role of these factors in the control of biofilm formation and adhesion to epithelial cells. Proteomic approaches could also be used with the aim to identify which proteins are expressed and modulated when local environmental conditions change.

In addition to the suggestions aforementioned, it is generally known that the use of indwelling medical devices is increasing and these are known risk factors in candidosis. Consequently further work could also investigate the role of intrauterine devices in enhancing *Candida* colonisation and biofilm formation, to further elucidate our understanding of VC.

In summary, this thesis has revealed the importance of studying natural plant extracts and associated individual components as a means to increase the number of available antifungal agents for the treatment of candidosis. In addition, inherent differences between *C. albicans* and *C. glabrata* RHVE pathogenicity were determined and the increase of *C. glabrata* pathogenicity in presence of *C. albicans* also demonstrated. Furthermore, it was also observed that the presence of progesterone decreased the capacity of *C. albicans* to form biofilm and colonise/invade the RHVE. Thus, this *in vitro* work provides potentially valuable information into the pathogenic mechanisms of *C. albicans* and NCAC species.