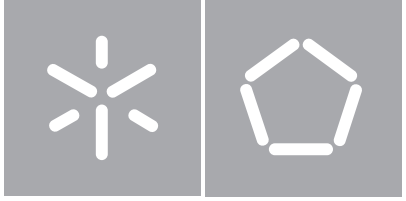

Universidade do Minho
Escola de Engenharia

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Insights into *Candida tropicalis* virulence factors

Dissertation for PhD degree in Biomedical Engineering

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Melyssa Fernanda Norman Negri Grassi

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“O que vale na vida não é o ponto de partida e sim a caminhada.
Caminhando e semeando, no fim terás o que colher.”

Cora Coralina

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Numa folha qualquer eu desenho um sol amarelo
E com cinco ou seis retas é fácil fazer um castelo.

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E se faço chover, com dois riscos tenho um guarda-chuva.

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Num instante imagino uma linda gaivota a voar no céu.

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Basta imaginar e ele está partindo, sereno, lindo,
E se a gente quiser ele vai pousar.

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E ali logo em frente, a esperar pela gente, o futuro está.

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O futuro nos espera e vamos ver no que vai dar...

Nessa estrada não nos cabe conhecer ou ver o que virá.
O fim dela ninguém sabe bem ao certo onde vai dar.
Vamos todos numa linda passarela.
De uma aquarela que um dia, enfim, descolorirá.

Numa folha qualquer eu desenho um sol amarelo (que descolorirá).
E com cinco ou seis retas é fácil fazer um castelo (que descolorirá).
Giro um simples compasso e num círculo eu faço o mundo (que descolorirá).

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Melyssa Negri

Abstract - Insights into *Candida tropicalis* virulence factors

Candida tropicalis is a common species related to nosocomial infections, namely candidemia and candiduria. Several virulence factors seem to be responsible for *C. tropicalis* infections, which present high potential for dissemination and mortality. Adhesion to surfaces (medical devices and host cells) and biofilm formation, are considered important factors that contribute to the development of candidosis. Hence, the colonization of indwelling devices like urinary catheters by *C. tropicalis* poses a critical problem. Further, adhesion and invasion of host cells by *C. tropicalis* is considered the first step to initiate systemic infections. Once adhered to epithelium, *C. tropicalis* are able to secrete hydrolytic enzymes that cause damage in host cells membrane integrity, leading to dysfunction or disruption of host structures. Thus, the main aim of this work was to characterize the virulence factors of *C. tropicalis* as well as to evaluate adhesion to biotic and abiotic surfaces, biofilm formation, expression of hydrolytic enzymes and antifungal susceptibility of *C. tropicalis* clinical isolates from urine and blood cultures and from central venous catheters.

Accordingly, in order to enhance the knowledge in the process of *C. tropicalis* adhesion and consequent biofilm formation in urinary catheters, the first goal of this research was to develop an *in vitro* dynamic model, with silicone and latex urinary catheters, using artificial urine (AU). Moreover, *Candida* surface hydrophobicity was also evaluated, as well as the biofilm matrix content in terms of proteins and carbohydrates. So, this model using AU was shown to be suitable for studies mimicking the real body conditions. Additionally, *C. tropicalis* was, in fact, able to colonize both urinary catheters in the presence of AU and to detach from these catheters and move against the flow, demonstrating their ability to colonize distal sites.

In vitro studies for the assessment of yeast cells adhesion capability to host tissues are essential to characterise the virulence of *Candida* species. However, the assessment of the number of adhered yeast cells by traditional methods is time consuming. Therefore, a simple methodology, using crystal violet staining, was developed to quantify *in vitro* adhesion of different *Candida* species to epithelial cells. The method was validated for the different *Candida* reference strains of different species by comparison with traditional microscope observation and enumeration. The proposed technique is easy to perform and reproducible, enabling the determination of adhesion ability of *Candida* species to an epithelial cell line.

After standardizing the methodologies to evaluate *Candida* adhesion ability, the next step was the characterization of *C. tropicalis* virulence, by assessing antifungal susceptibility and comparing the expression of several virulence factors. Regarding adhesion, it can be highlighted that *C. tropicalis* strains adhered in significantly higher number to epithelium than to silicone. Furthermore, all *C. tropicalis* strains were able to form biofilms and to express total haemolytic activity. However, protease and phospholipase positive response were detected only in few isolates but from different sites of isolation. All isolates were susceptible to voriconazole, fluconazole and amphotericin B. Four strains were

susceptible-dose dependent to itraconazole and one clinical isolate was found to be resistant to this agent.

Then, it was investigated the interaction of *C. tropicalis* with three different human cell lines: TCC-SUP (epithelial cells from urinary bladder); HeLa (epithelial cells from cervical carcinoma) and Caco-2 (epithelial cells from colorectal adenocarcinoma). Specifically, the degree of human cells damage and activity reduction induced by *C. tropicalis* adhesion and the role of *Candida tropicalis* aspartyl proteinases (*SAPT*) genes expression in this process were assessed. It was possible to observed that *C. tropicalis* strains were able to adhere to the different human cells, although, in a strain and cell dependent manner. Concerning human cells response to *C. tropicalis*, the highest cell activity inhibition was obtained for Caco-2, followed by TCC-SUP and HeLa cells. *C. tropicalis* strains in contact with the different types of epithelial cells exhibited a wide range of expression profiles of *SAPT* genes, however, *SAPT3* was the gene expressed in a higher level.

Finally, it was studied the behaviour of *C. tropicalis* in biofilms of different ages (24-120 h) formed in artificial urine (AU) and their effect in human urinary bladder cells (TCC-SUP). A similar profile in metabolic activity along biofilm age was found among strains, with an increase from 72 to 96 h and a decrease from 96 to 120 h. *Candida tropicalis* biofilm cells were able to adhere to TCC-SUP cells, in general, independently of biofilm age. Yeasts affected TCC-SUP cells, with difference among biofilms and strains. Generally, *SAPT3* was highly expressed in comparison with other *SAPT* genes.

In summary, *C. tropicalis* strains were able to form biofilms in AU, in static or dynamic mode, although, with differences among strains. It is important to emphasize that human cells response to *C. tropicalis* adhesion, as well as *SAPs* production, is strain and epithelial cell line dependent. Additionally, it should be highlighted that *C. tropicalis* cells detached from biofilms are able to colonize human cells and cause some injury and reduction of metabolic activity. Generally, *SAPT3* was highly expressed compared to other *SAPT* genes.

Resumo - Fatores de virulência de *Candida tropicalis*

Candida tropicalis é uma espécie comummente relacionada com infecções nosocomiais, tais como, candidemia e candidúria. Vários fatores de virulência parecem ser responsáveis por infecções por *C. tropicalis*, que apresentam elevado potencial de disseminação e mortalidade. A adesão às superfícies (dispositivos médicos e células do hospedeiro) e a formação de biofilmes, são considerados factores importantes que contribuem para o desenvolvimento de candidose. Assim, a colonização do interior de cateteres urinários por *C. tropicalis* representa um problema crítico. Além disso, adesão e invasão das células hospedeiras por *C. tropicalis* é considerado o primeiro passo para iniciar infecções sistémicas. Uma vez aderidas ao epitélio, as células de *C. tropicalis* são capazes de excretar enzimas hidrolíticas que causam danos da membrana de células do hospedeiro. Assim, o objetivo principal deste trabalho foi caracterizar os factores de virulência de *C. tropicalis*, incluindo a avaliação da adesão às superfícies bióticas e abióticas, formação de biofilme, a expressão de enzimas hidrolíticas e suscetibilidade aos antifúngicos

Assim, a fim de aumentar o conhecimento no processo de adesão de *C. tropicalis* e consequente formação de biofilme em cateteres urinários, o primeiro objetivo deste trabalho foi desenvolver um modelo dinâmico *in vitro*, com cateteres urinários de silicone e látex, com urina artificial (UA). Além disso, hidrofobicidade superficial de *Candida* também foi avaliada, assim como o conteúdo da matriz do biofilme, em termos de proteínas e hidratos de carbono. Assim, este modelo mostrou-se adequado para estudos simulando as condições reais do corpo. Além disso, *C. tropicalis* foi, de facto, capaz de colonizar os cateteres urinários na presença de UA e destacar a partir desses cateteres e mover contra o fluxo imposto, demonstrando sua capacidade de colonizar locais mais distais.

Apesar de ser fundamental desenvolver estudos *in vitro* para a avaliação da capacidade de adesão de leveduras aos tecidos, a avaliação do número de células de leveduras aderidas por métodos tradicionais é demorada. Assim tornou-se necessário desenvolver uma metodologia simples, utilizando uma coloração com violeta cristal para quantificar a adesão *in vitro* de diferentes espécies de *Candida* a células epiteliais. O método foi validado para diferentes espécies de *Candida* e foi feita a comparação com a enumeração por observação ao microscópio. A técnica proposta é de fácil execução e reprodutível, permitindo a determinação da capacidade de adesão das espécies de *Candida* a uma linha de células epiteliais.

Um outro objetivo do presente trabalho foi a caracterização da virulência de *C. tropicalis*, através da avaliação da susceptibilidade aos antifúngicos e comparação com a expressão de factores de virulência. Verificou-se que as estirpes de *C. tropicalis* aderiram em número significativamente superior ao epitélio do que ao silicone, foram capazes de formar biofilmes e de manifestar atividade hemolítica total. No entanto, a protease e a fosfolipase foram detectadas apenas em alguns isolados. Todos os isolados foram susceptíveis ao voriconazol, fluconazol e anfotericina B. Quatro estirpes foram susceptíveis dose dependente ao itraconazol e um isolado clínico foi resistente a este agente.

Em seguida, foi investigada a interação de *C. tropicalis* com três linhas celulares humanas diferentes: TCC-SUP (células epiteliais da bexiga); HeLa (células epiteliais de carcinoma do colo do útero) e Caco-2 (células epiteliais do adenocarcinoma colorretal). Especificamente, foram avaliados o grau de lesão das células humanas induzida por *C. tropicalis* e o papel da expressão do gene aspartil protease (*SAPT*), neste processo. Foi possível observar que as estirpes de *C. tropicalis* foram capazes de aderir às diferentes células humanas, embora de forma dependente da linha celular e da estirpe. Quanto à resposta de células humanas, verificou-se uma maior inibição de atividade celular em Caco-2, seguido de TCC-SUP e HeLa. As estirpes de *C. tropicalis* em contato com os diferentes tipos de células epiteliais apresentaram uma ampla variedade de perfis de expressão de genes *SAPT*, no entanto, *SAPT3* foi o gene expresso em maior quantidade.

Por fim, foi estudado o efeito de biofilmes de *C. tropicalis* (24-120 h), formados em UA, em células TCC-SUP. Foi então detetado um perfil semelhante na atividade metabólica dos biofilmes das diferentes estirpes, com um aumento das 72 h para as 96 h, e uma diminuição das 96h para as 120 h. De um modo geral, as células de *C. tropicalis* provenientes dos biofilmes foram capazes de aderir a células TCC-SUP, independentemente da idade do biofilme. As leveduras afetaram as células TCC-SUP, com diferenças entre os biofilmes e as estirpes. Em geral, o gene *SAPT3* foi mais expresso em comparação com outros genes *SAPT*.

Em resumo, as estirpes de *C. tropicalis* estudadas foram capazes de formar biofilmes na UA, no modo estático ou dinâmico, embora com diferenças entre as estirpes. É importante ressaltar que a resposta de células humanas para à adesão *C. tropicalis*, bem como a produção de *SAPT*s, é dependente da estirpe e da linha celular. Além disso, deve-se ressaltar que as células de *C. tropicalis* isoladas de biofilmes são capazes de colonizar as células humanas e causar alguma lesão e redução da atividade metabólica. Em geral, o gene *SAPT3* foi o mais expresso.

Scope and outline of thesis

Usually, *Candida tropicalis* is considered the first or the second non-*Candida albicans Candida* (NCAC) species most frequently isolated from bloodstream (candidemia) and from urinary tract (candiduria). Additionally, *C. tropicalis* is often found in patients admitted to Intensive Care Units (ICU), especially in patients with cancer, requiring prolonged catheterization, or/and receiving broad-spectrum antibiotics

Several virulence factors seem to be responsible for *C. tropicalis* infections, which present high potential for dissemination and mortality. Adhesion to abiotic surfaces (medical devices) or to host tissues, as well as biofilm formation, secretion of enzymes (proteases and phospholipases) and haemolytic activity are considered important factors in *C. tropicalis* mechanisms of infection. Therefore, the need to get more insights in *C. tropicalis* virulence was the driving force for the research performed.

The present thesis reports the works totally carried out in the Laboratory of Applied Microbiology – Biofilm Group, at IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Braga, Portugal.

For the development of this research, several samples of *Candida tropicalis* were used, and were clinical isolates from patients admitted to the ICU of University Hospital in Maringá, kindly donated by Terezinha Svidzinski, Division of Medical Mycology, Universidade Estadual de Maringá, Paraná, Brazil. Previously, these strains were already used, by Melyssa Negri, when preparing her master thesis, and relevant information about these strains is found in Table 1.

Table 1: Characteristics of *Candida tropicalis* strains used in the present thesis

Code	Site of isolation	Candidosis	Age	Sex	Intensive Care Unit
S01	blood	candidemia	45	female	oncologic
U12	urine	candiduria	86	female	-
U16	urine	candiduria	69	female	-
U29	urine	candiduria	80	female	-
U69	urine	candiduria	84	female	oncologic
U75	Urine	candiduria	28	female	-
CL012	central venous catheter	candidemia	39	male	-

The present thesis is organized in seven Chapters, reporting studies, which address the main mechanisms related to *Candida tropicalis* pathogenesis. So, **Chapter 1 - "Synopsis of *Candida tropicalis*: nosocomial infection and virulence factors"** is a brief review on the relevant aspects of *C. tropicalis* biology and summarizes the present knowledge on *C. tropicalis* pathogenesis.

To better understand the ability of *C. tropicalis* to infect through biofilm formation in urinary catheters, it was necessary to assess how *C. tropicalis* form biofilms in a system mimicking the real situation. Thus, in **Chapter 2 - "*Candida tropicalis* biofilms: artificial urine, urinary catheters and flow model",** is described a model to study the ability of *C. tropicalis* to form biofilm using artificial urine (AU) and urinary catheters, under flow conditions.

Since *Candida* species are able to detach from biofilms and colonize others sites such as host cells, another point addressed in this work was the development of a technique easy to perform and reproducible for the assessment of *Candida* species ability to adhere to an epithelial cell line, which is described in **Chapter 3 - "Crystal violet staining to quantify *Candida* adhesion to epithelial cells".**

After that optimization step and also contributing to deepen the knowledge on *C. tropicalis* virulence factors, next **Chapter 4 - "Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients"** is focused on the characterization of *C. tropicalis* virulence by assessing the susceptibility to the most common antifungal agents and comparing the expression of several virulence factors.

The last studies performed under the scope of this thesis were directed to investigate the interaction of *C. tropicalis* with human cells. In particular, **Chapter 5** - "**An *in vitro* evaluation of *Candida tropicalis* infectivity using human cell monolayers"** describes the degree of human cells damage and their activity reduction induced by *C. tropicalis* adhesion to different human epithelial cell lines and the role of *SAPT* gene expression in this process. **Chapter 6** "***Candida tropicalis* biofilms: effect on urinary epithelial cells"** reports the behaviour of *C. tropicalis* in biofilms of different ages (24 – 120 h) formed in AU and their effect on human urinary bladder cells (TCC-SUP).

Finally, **Chapter 7 - "Concluding remarks and future perspectives"** highlights the main conclusions, obtained in this thesis, concerning *Candida tropicalis* and its virulence factors and proposes suggestions for future research that can contribute for enhanced understanding of *C. tropicalis* pathogenesis.

Publications within the thesis

PAPERS IN PEER REVIEWED JOURNALS

Published

1. **Negri M**, Henriques M, Svidzinski TIE, Paula CR, Oliveira R. Correlation between Etest®, disk diffusion, and microdilution methods for antifungal susceptibility testing of *Candida* species from infection and colonization. J Clin Lab Anal. 2009; 23 (5): 324-30.
2. **Negri M**, Gonçalves V, Silva S, Henriques M, Azeredo J, Oliveira R. Crystal violet staining to quantify *Candida* adhesion to epithelial cells. Br J Microbiol 2010; 67 (3): 120-5. (**Chapter 3**)
3. **Negri M**, Martins M, Henriques M, Svidzinski T, Azeredo J, Oliveira R. Examination of Potential Virulence Factors of *Candida tropicalis* Clinical Isolates From Hospitalized Patients. Mycopathologia. 2010, 169 (3): 175-82. (**Chapter 4**)
4. Silva S, **Negri M**, Henriques M, Oliveira R, Williams D, Azeredo J. Silicone colonization by non-*Candida albicans Candida* species in the presence of urine. J Med Microbiol. 2010; 59 (7): 747-54.
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Submitted

1. **Negri M**, Silva S, Henriques M, Azeredo J, Oliveira R. *Candida tropicalis* biofilms: effect on urinary epithelial cells. (**Chapter 6**)
2. **Negri M**, Silva S, Henriques M, Oliveira R. Synopsis of *Candida tropicalis*: the role in nosocomial infections and virulence factors. (**Chapter 1**)
3. Monteiro DR, Gorup LF, Silva S, **Negri M**, Camargo ER, Oliveira R, Barbosa DB, Henriques M. Silver colloidal nanoparticles: antifungal effect against *Candida albicans* and *Candida glabrata* adhered cells and biofilms.
4. Lourenço T, Silva S, **Negri M**, Henriques M, Azeredo J, Oliveira R. *Candida glabrata* antifungal susceptibility: planktonic, biofilms and biofilm extracellular matrix composition.

OTHERS SCIENTIFIC OUTPUT

Book chapter

1. **Negri M**, Lorenço T, Silva S, Henriques M, Azeredo J, Oliveira R. Effect of antifungal agents on Non-*Candida albicans Candida* species enzymes secretion. 2011, p. 313-317. Book title: "Science and Technology against Microbial Pathogens. Research, Development and Evaluation". ISBN-13: 978-981-4354-85-1
2. Henriques M, **Negri M**, Silva S. 2011. O impacto clínico de biofilmes de espécies de *Candida*.

Oral presentation

1. **Negri M**, Brêda D, Henriques M, Azeredo J, Oliveira R. The role of *Candida tropicalis* biofilms on human urinary bladder cells. Biofilms in Nosocomial Fungal Infections, Paris, 31 January-1 February, 2011. Book of Abstracts. O8, pag. 37.

Posters in conferences

2. Negri MF, Ribeiro A., Svidzinski TI, Henriques M., Oliveira R. Correlation between e-test, disk diffusion, and microdilution methods for antifungal susceptibility testing of *Candida* species**.** 9th Candida and Candidiasis, New York, March 24-28, 2008. Book of Abstracts. B 269. page 126.
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11. Botelho C, **Negri M**, Silva S, Henriques M, Azeredo J, Oliveira R. Adhesion of non-*Candida albicans Candida* spp to urinary epithelial cells. 10^{th} ASM Conference on Candida and Candidiasis, Miami, March 22^{nd} – 26^{th}, 2010. Book of Abstracts. 174B. page 125.
12. **Negri M**, Botelho C, Silva S, Henriques M, Svidzinski T, Azeredo J, Oliveira R. *Candida tropicalis* biofilms: formation and virulence factors. Biofilms 4 International. Conference, Winchester, September 2 – 3, 2010. Book of Abstracts. 28. page 37.
13. **Negri M**, Lorenço T, Silva S, Henriques M, Azeredo J, Oliveira R. Effect of antifungal agents on Non-*Candida albicans Candida* species enzymes secretion. International Conference on Antimicrobial Research ICAR, Valladolid, November 3rd – 5th, 2010. Book of Abstracts. F 84. page 392.
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17. Monteiro DR, Silva SC, **Negri M**, Camargo ER, Oliveira R, Henriques M, Barbosa DB. Effect of silver nanoparticles against Candida albicans and Candida glabrata biofilms. Biofilms in Nosocomial Fungal Infections, Paris, 31 January-1 February, 2011. Book of Abstracts. page 91.

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Abbreviation and acronyms

P: Significance value

g: Gravity

Θ: Water contact angle (^{o})

χ^{+}: Electron acceptor surface tension parameter (mJ/m^2)

χ^{-}: Electron donor surface tension parameter (mJ/m^2)

ΔG_{sws}: Total free energy variation between entities of a given surface(s) immersed in water (w) (mJ/m^2)

%: Percent

ΔC_T: Threshold cycle

°C: Degrees Celsius

h: Hour

l: Liter

ml: Milliliter

nm: nanometer

µg: Microgram

µm: Micrometer

µM: Micromolar

λ: wavelength

Abs: Absorbance

ALS: Agglutinin like sequence gene

Als: Agglutinin like sequence protein

ANOVA-Analysis of variance

ATCC: American Type Culture Collection

AU: Artificial urine

BSA: Bovine Serum Albumin

CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

cDNA: complementary Deoxyribonucleic Acid

CFU: Colony Forming Units

CHROMagar-Chromogenic media agar

CLSM: Confocal Laser Scanning Microscopy

CV: Crystal violet

DNA: Deoxyribonucleic Acid

FCT: Fundação para a Ciência e Tecnologia

LDH: Lactate Dehydrogenase

Log: Logarithm

min: Minute

mRNA: messenger Ribonucleic Acid

MTS: ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H–tetrazolium]

NCAC: non-*Candida albicans Candida*

ND-No detected

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PLs: Phospholipases

RHOE: Reconstituted Human Oral Epithelium

RNA: Ribonucleic Acid

Rpm: rotation per minute

rRNA: ribosomal Ribonucleic Acid

SAP: Secreted aspartly proteinase gene

Sap: Secreted aspartly proteinase protein

SDA: Sabouraud dextrose agar

SDB: Sabouraud dextrose broth

SD: Standard deviation

SEM: Scanning Electron Microscopy

SPSS: Statistical package for the social sciences

UA: Urina artificial

UTIs: Urinary Tract Infections

U: Units

v: Volume

V: Voltage

w: Weight

XTT: 2, 3 bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]2-Htetrazolium hydroxide

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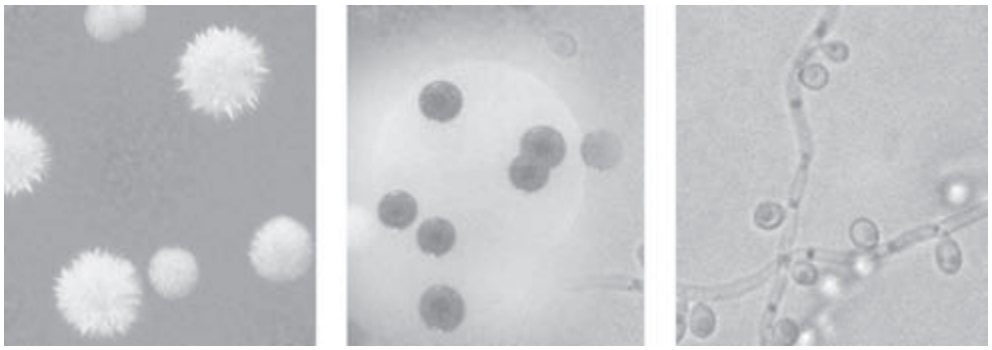
CHAPTER 1

Synopsis of *Candida tropicalis*: the role in nosocomial infections and virulence factors



ABSTRACT

Candida tropicalis is considered the first or the second non-*Candida albicans Candida* (NCAC) species most frequently isolated from candidosis, mainly in patients admitted in intensive care units (ICUs), specially with cancer, requiring prolonged catheterization, or receiving broad-spectrum antibiotics. The proportion of candiduria and candidemia caused by *C. tropicalis* varies widely with geographical area and patient group. Actually, in certain countries, *C. tropicalis* is more prevalent, even compared with *C. albicans* or other NCAC species. Although prophylactic treatments with fluconazole cause a decrease in the frequency of candidosis caused by *C. tropicalis,* on other hand, *C. tropicalis* is increasingly showing a moderate level of fluconazole resistance. The propensity of *C. tropicalis* for dissemination and the high mortality associated to its infections might be strongly related to the potential of virulence factors exhibited by this species, such as adhesion to different host surfaces; biofilm formation; infection and dissemination; and enzymes secretion. Therefore, the aim of this review is to outline the present knowledge on all the above mention *C. tropicalis* virulence traits.

Keywords: *Candida tropicalis*; epidemiology; risk factors; virulence factors; candiduria; candidemia.

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INTRODUCTION

Nosocomial infections (NIs), or in other words hospital acquired infections, are now a serious public health problem, since these infections are among the leading causes of morbidity and mortality, causing an increase in hospitalization time and, consequently, high costs associated to patient´s treatment [1, 2]. NIs have been particularly prominent in intensive care units (ICUs), where the incidence is two to five times higher than in the general population of hospitalized patients [3, 4]. The causes for the increased risk of NIs in ICUs have been associated with increased length of stay in ICU, invasive procedures, patients with compromised immune systems, and multiple exposure to antibiotics [5-7]. Beyond the hospital unit and the disease involving the patient, factors related to the infecting organism are of major importance to the progression of hospital acquired infections [8]. Most of the NIs is caused by microorganisms of the normal microbiota that attack the patient in special situations like under immunosupression. In these patients, considered at risk, invasive fungal infections are often severe, with a rapid progression and difficult to diagnose and/or treat [1, 7].

Fungal nosocomial infections (FNIs) incidence has increased significantly over the last decades. *Candida* species are the most frequently isolated fungi, corresponding to approximately 80% of FNIs, being the fourth responsible for blood stream infections and the overwhelming majority being responsible for urinary tract infections [7, 9, 10].

Until some years ago, *Candida albicans* was the *Candida* species that received major clinical attention. However, in parallel with the overall increase of fungal infections, it has been observed that infections caused by non-*Candida albicans Candida* (NCAC) species are emerging [7, 11, 12]. The reasons for this alteration in the pattern of *Candida* species distribution has not yet been completely understood, but could be attributed to the resistance of the NCAC species to antifungal agents, which are used for relatively long periods during hospitalisation [9, 12-14].

Usually, *Candida tropicalis* is considered the first or the second NCAC species most frequently isolated from bloodstream (candidemia) [12, 13, 15, 16] and from urinary tract (candiduria) [17, 18] infections. Additionally, *C. tropicalis* is often found in patients admitted to ICUs, especially in patients with cancer, requiring prolonged catheterization, and/or receiving broad-spectrum antibiotics [8, 12]. This species appears to display higher potential for dissemination in the neutropenic host than *C. albicans* and other NCAC species. This propensity for dissemination in some way may explain the reported relatively high mortality associated with *C. tropicalis* [15, 19, 20].

Several virulence factors seem to be responsible for *C. tropicalis* infections, which present high potential for dissemination and mortality [21]. Adhesion to host surfaces (epithelial cells and medical devices), as well as biofilm formation [22, 23], secretion of enzymes (proteinases and phospholipases) and haemolytic activity are considered important factors in *C. tropicalis* infection [22, 24, 25]. Therefore, this article aims to review and discuss *C. tropicalis* general characteristics, focusing on its microbiology, epidemioogy, risk factors and mainly on its virulence factors.

MICROBIOLOGY

Candida tropicalis, firstly known as *Oidium tropicale,* was differentiated among several *Candida* species in 1910 by Aldo Castellani. Meanwhile other names have been attributed to this species, as *Monilia tropicalis*, *Candida vulgaris*, *Mycotorula dimorpha*, *Candida paratropicalis* and other 58 synonyms. Only in 1923, Berkhout introduced the present name [26, 27]. *Candida tropicalis* is a diploid ascomycete yeast and an opportunistic human pathogen, which colonizes several anatomically distinct sites, including the skin [28, 29], gastrointestinal [30] and genitourinary tracts [28], and may also be seen in the respiratory tract [29]. It can also be recovered from the environment, particularly from surfaces in medical setting [22, 29, 31]. Moreover, since 1960 *C. tropicalis* has been recognized as responsible for serious invasive candidosis [32, 33].

Infections caused by *C. tropicalis* can be acquired endogenously, when the individual is already colonized by the microorganism as part of the normal flora, but under altered conditions yeasts may be translocated and spread through the gastrointestinal tract to different anatomic sites, causing infection [8, 12, 15]. The exogenous infection can occur through contact of the hands of health professionals with patients or through catheters, implantable prostheses, as well as parenteral solutions, which were previously contaminated [15, 22, 34, 35].

The mechanism used by the commensal *C. tropicalis* to become a human pathogen is not yet clear. Moreover *C. tropicalis* infections involve a broad spectrum of invasive diseases, affecting patients exposed to wide variety of risk factors [8, 36, 37]. Among the invasive infections caused by *C. tropicalis*, the most common are candiduria and candidemia [13, 15, 17, 18, 38].

IDENTIFICATION

Colonies of *C. tropicalis* are cream-colored with a slightly mycelial border (Figure 1.1 a) on the routinely used Sabouraud dextrose agar (SDA) and appear dark blue (Figure 1.1 b) in *CHROMagar™ Candida* (CHROMagar, Paris, France) [26, 39]. Microscopically (Figure 1.1 c), on corn meal Tween 80 agar at 25 °C (Dalmau method), *C. tropicalis* shows blastoconidia singly or in small groups all along graceful, long pseudohyphae and may also produce true hyphae.

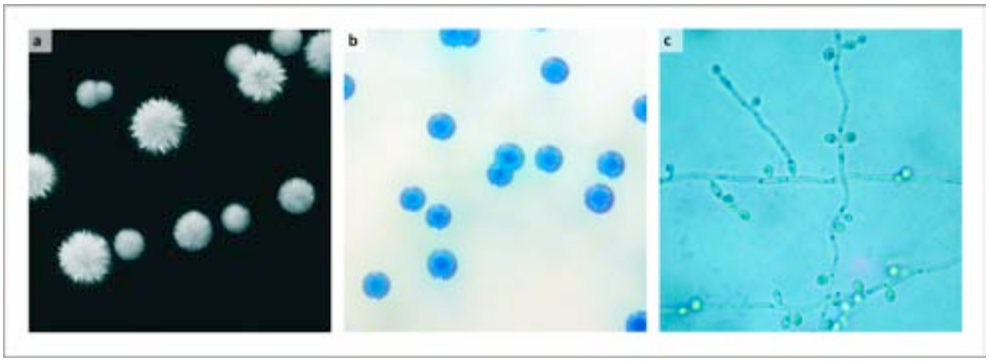

Figure 1.1: *Candida tropicalis* morphology in routinely culture media: a) Colonies of *C. tropicalis* on Sabouraud dextrose agar; b) on *CHROMagar™ Candida*; c) on corn meal Tween 80 agar.

In biochemical tests (fermentation and assimilation, Table 1.1), this yeast differs from the other important *Candida* species by being able to ferment and to assimilate glucose, sucrose, galactose, trehalose, and maltose, but not lactose or rafkose [26, 40, 41].

Table 1.1: Microbiological and biochemical characteristics of *C. tropicalis* compared with other important *Candida* species

Test		*Candida* species			
		C. tropicalis	*C. albicans*	*C. parapsilosis*	*C. glabrata*
Microbiology					
	hyphae	+	+	-	-
	pseudohyphae	+	+	+	-
	germinative tube	-	+	-	-
*Biochemical					
	glucose	FA	FA	AF±	FA
	galactose	FA	AF±	A	-
	lactose	-	-	-	-
	maltose	FA	FA	A	-
	sucrose	FA	A	AF±	-
	melibiose	-	-	-	-
	celobiose	A±	-	-	-
	trehalose	FA	AF±	AF±	A±
	raffinose	-	-	-	-
	melezitose	A	A±	A	-
	urease	-	-	-	-
	KHO_3	-	-	-	-

*Biochemical analyses: Fermentation and assimilation in the presence of carbon source. Urea hydrolysis and assimilation of KHO_3. (+) Positive; (-) negative; (FA) fermentation and assimilation positive; (A) assimilation positive; (A±) assimilation variable; assimilation positive with fermentation variable (AF±).

Genetically, *C. tropicalis* is more similar to *C. albicans*, since it contains the major repeat sequence (MRS) elements, than *C. parapsilosis* and *C. glabrata* [42]. The discovery of MRS elements in *C. tropicalis* suggests that these repeats play a similar role in karyotypic variation in this species, although the contribution of these changes to pathogenesis is not known [43, 44].

For molecular identification, several procedures have been proposed to detect and differentiate *Candida* species *in vitro*, either by DNA extraction from cultured organisms [45, 46] or directly from clinical samples [47-49]. Methods as polymerase chain reaction (PCR) assay [47, 49, 50] and real-time PCR assays [45, 51], described in Table 1.2, have been successful used to identify *C. tropicalis* from clinical samples and even when this species is found in the presence of other fungi. Nevertheless, these methodologies

are not yet standardized or readily available in most clinical laboratory settings nor have been validated in large clinical trials.

Table 1.2: Primers and probes used for polymerase chain reaction (PCR) and real-time PCR assay used for the identification of *C. tropicalis* from clinical samples and when this species is found in the presence of other fungi

Molecular method (reference)	Sequence (direction)	Description
PCR-based [47]	*C. tropicalis* I (F) 5′-GTTGTACAAGCAGACATGGACTG-3′ (R) 5′-CAAGGTGCCGTCTTCGGCTAAT-3′ (R) 5′-TCAAGGTACAGTTATGGCCAAGTT-3′ *C. tropicalis* II (F) 5′-CTGGGAAATTATATAAGCAAGTT-3′ (R) 5′-CTTGAGATACTCAATCTTTTATC-3′ (R) 5′-TCAATGTACAATTATGACCGAGTT-3′	Primer mixes specific to *Candida* DNA topoisomerase II genes. For the identification of *Candida tropicalis* to the species level, one species-specific forward primer and two species-specific reverse primers were designed within the region amplified by the degenerated primer pair.
Multiplex PCR [50, 52]	ITS1 5′-TCC GTA GGT GAA CCT GCG G-3′ ITS2 5′-GCT GCG TTC TTC ATC GAT CG-3′	The method is based on the size variability of the ITS1 region in different species. The fungi-specific primers ITS1 and ITS2 are used to amplify a small conserved portion of the 18S rDNA region, the adjacent ITS1, and a small portion of the 28S rDNA region, generating different PCR products for *C. glabrata*, *C. guilliermondii*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*.
PCR and pyrosequencing [49]	PCR: bio-fun (F) 5′-Biotin-ATTGGAGGGCAAGTCTGGTG-3′ fun (R) 5′-CCGATCCCTAGTCGGCAT-3′ Pyrosequencing: funS 5′-YTCAMAGTAAAAGTCCTGG-3′ or funS2 5′-TCAAAGTAAAAGTCCTGGTTC-3′ *C. tropicalis* pyrosequencing with primers funS or funS2: TTCGCCAAAAGGCTAGCCAGAAGGAAAGGC TCGGTTGGGTC	The tests are performed on amplicons derived from the 18S rRNA gene using PCR universal primers for amplification. The amplification products were subjected to pyrosequencing analysis - a method of DNA sequencing (determining the order of nucleotides in DNA) based on the "sequencing by synthesis" principle.
LightCycler PCR [45, 53]	Primer CTR-PR (F) 5′-TCATACCAGTGATAGATGG-3′ CTR-PR (R) 5′-TTTTCTAGCTACTCCATGG-3′ Probes CTR-FL 5′-GTTGATTACCAATCCATGGTTACCTTAC-3′ CTR-RED 5′-ATTAGAACCTGCTGAAAT**T**GTTTGG-3′	The LightCycler PCR combines rapid amplification of nucleic acids in glass capillary with melting curve analysis based on fluorescence resonance energy transfer for the sensitive detection of point mutations in various settings. Species-specific amplification (standard PCR) and hybridization (LightCycler PCR) of *Candida* DNA could be achieved using the species-specific primer pairs and the oligonucleotides, respectively.
Real-time PCR assays [48]	*Candida*-specific primers Cand (F) CCTGTTTGAGCGTCRTTT ITS (R) TCCTCCGCTTATTGATAT *Candida*-specific probes C.trop-S Cy5-GGCCACCACAATTTATTTCA-biotin	The application of the biprobe technology facilitated a rapid screening for fungi (specific for the fungal ITS2 region) and simultaneous differentiation of 11 medically important *Aspergillus* and *Candida* species (with species-specific biprobes) in only two individual PCR mixtures and simultaneously in the same LightCycler run.

(F) and (R) indicate forward and reverse primers, respectively.

RISK FACTORS

In general, the risk factors involved in the development of hospital-acquired *Candida* infections are associated with: extended periods in intensive care unit, administration of broad-spectrum antibiotics, patients with immunosuppression, indwelling catheters, mechanical ventilation, candiduria, multiple sites of colonization, burns, and haemodialysis [5, 54-56]. However, the particularities of each *Candida* species may be influenced by specific risk factors. Studies have been shown, that in opposition to *C. parapsilosis*, *C. tropicalis* was less likely to occur among children of less than 1 year of age, but more likely to occur in patients with cancer or neutropenia [15, 57, 58], and is strongly associated with the presence of biofilms in urinary catheters. [18, 54, 59-61].

Candida colonization remains the most universally accepted predictive variable with regard to invasive candidosis, being particularly true for high density colonization. In fact, colonization by *C. tropicalis,* especially from a specific body site can be highly predictive of the development of invasive disease with this organism [62]. Nevertheless it has not yet been clarified whether colonization can be used alone to identify high-risk patients or if it should be combined with other variables indicating high risk [63]. According to Paul *et al*. [64] many risk factors traditionally linked to candiduria may be associated with urinary tract infections in general. Furthermore, Binelli *et al.* [65] found a significant association of candidemia with candiduria, although urine was not the main source of *C. tropicalis* bloodstream infection.

According to epidemiological data, when comparing patients with candidemia caused by *C. tropicalis* to those caused by other species of *Candida*, the former are, in average, older patients (67 years vs. 56 years, $P = 0.01$), present cancer (45.5% vs. 31.6%, $P = 0.04$), and that the portal of entry is the abdomen (32.2% vs. 11.9%, $P = 0.001$). Additionally, these patients also have a high hospital mortality rate (61% vs. 44%, $P = 0.03$) [66]. Further studies suggested that *C. tropicalis* is associated with higher dissemination potential and mortality in patients admitted in ICU,

particularly in oncology patients, than *C. albicans* or any other NCAC species [13, 15, 67].

EPIDEMIOLOGY

The proportion of candidoses (candidemia and candiduria) caused by *C. tropicalis* varies widely with geographical area and patient group, with *C. tropicalis* being more prevalent, even compared with *C. albicans,* in certain countries [66, 68, 69]. Considering Table 1.3, it is possible to see that, among NCAC species, *C. tropicalis* has been considered the species most frequently isolated from candidosis in the Pacific-Asia region [13], Brazil [12, 15], and recently in Europe [7, 70]. Furthermore, important epidemiological studies revealed that 90% of invasive candidosis were due to NCAC species, with *C. tropicalis* accounting for about 4.6% in 1997-1998; 5.3% in 1999; and between 7.3% in 2000-2003 [71]. Additionally, in general, *C. tropicalis* appeared to be the first or second NCAC most frequently associated to candiduria. Nevertheless, regarding candidemia, *C. tropicalis* is less frequently isolated than *C. glabrata* [7, 17, 54, 67, 70].

Although the reasons for the increased detection of *C. tropicalis* in human infection are not completely clear the advent of molecular genetics, and the development of new methods of *Candida* identification and differentiation [45, 47, 72] may play an important role. Further, the changes of *C. tropicalis* incidence may also be attributed to the greater use of fluconazole nowadays [66, 69]. In the United States, due to fluconazole prophylaxis the frequency of candidosis caused for *C. tropicalis* has decreased. However, in other countries where it is not usual to use fluconazole prophylaxis, *C. tropicalis* appears more prevalent, e.g. second in Latin America, and even more common than *C. glabrata* in the Asia-Pacific region [71]. Nevertheless, the use of prophylactic fluconazole can become a risk factor since some cross-resistance between azoles [7, 11, 73] has been reported already. Several studies indicate that *C. tropicalis* has been showing a moderate level of fluconazole tolerance leading to the

need of an increase in the drug concentrations and enabling a risk of azole resistance [67, 74, 75].

Curiously, according to Table 1.3, the epidemiological data related with antifungal resistance, have been indicating an increase of *C. tropicalis* resistance to 5-flucytosine [12, 15, 54, 76, 77]. Furthermore, it was observed 35% of resistance to 5-flucytosine by *C. tropicalis* isolates recovered from blood cultures in the active surveillance program on yeast-related fungemia implemented by the French National Reference Center for Mycoses and Antifungals (NRCMA) in the Paris area [76]. Additionally, Densos-Olliver *et al*. [76] studied the relationship between epidemiologic and genomic data of *C. tropicalis* 5-flucytosine resistance and, observed that a clone of 5-flucytosine-resistant isolate, associated with malignancies, had lower mortality than the other *C. tropicalis* isolates. This suggests that geographic and temporal distribution of *C. topicalis* may be related with 5-flucytosine-resistant isolates in the Paris area.

In fact, the major problem with the development of invasive candidosis by *C. tropicalis* is that it is associated with higher mortality than other NCAC species and *C. albicans* [8, 20, 68, 78]. This propensity of *C. tropicalis* for dissemination and associated high mortality may be related to the virulence factors exhibited by this species such as biofilm formation; proteinases secretion and dissemination [11, 22].

Table 1.3: Summary of incidence and antifungal resistance attributed to *Candida tropicalis* candidosis (candidemia and candiduria)

Candidosis	References	Region/Country (period)	Number of strains	*C. tropicalis* (%)	*Other NCAC species (%)	Resistance (%)
Candidemia	[79]	Kuwait (1996-2005)	607	12.4	36.2	Flu (0) Itra (0) Vor (0.5) 5Flu (9.3)
	[80]	Europe (1997-1999)	2089	7	44	ND
	[77]	Italy (2000-2003)	94	16.0	35.1	Flu (0) Itra (0) Vor (0.5) 5Flu (3)
	[15]	Brazil (2003-2006)	924	20	20	Flu (6.6) Itra (6.6) Vor (0.5) 5Flu (20) Pos (6.6)
	[12]	Brazil (2003-2004)	712	20.9	25.4	Flu (0) Itra (0) Vor (0) 5Flu (5)
	[13]	India (2007)	140	42.1	6.4	Flu (10.2) Itra (13.6) Vor (10.2) Cas (2)
	[81]	Europe/Asia/ America (2008-2009)	1239	9.8	34.8	Flu (3.3) Vor (3.3) Pos (0.8) Cas (0)
Candiduria	[18]	USA (1991-1993)	530	7.9	19.7	ND
	[17]	Spain (1998-1999)	389	36	8.2	ND
	[65]	Brazil (1996-2000)	23	43.5	4.3	ND
	[82]	Slovakia	94	6.3	24.7	ND
	**[54]	France (2001-2002)	233	6.5	30.5	Flu (0) Itra (0) Vor (0) 5Flu (59) Cas (8.7)
	[70]	Portugal (2003-2006)	260	12.7	12.3	ND
	[75]	Brazil (2006-2007)	70	15.7	18.5	Flu (0) Itra (18.1)

Fluconazole (Flu); Itraconazole (Itra); Voriconazole (Vor); 5-Flucytosine (5Flu); Posoconazole (Pos); Caspofungin (Cas). Not determined (ND). * Percentage of *Candida glabrata* or/and *Candida parapsilosis.* ** *In vitro* susceptibilities of 22 *C. tropicalis* bloodstream and urine isolates

VIRULENCE FACTORS

Mechanisms used by *Candida* species with the purpose to cause any type of injury to the host are related with virulence factors. Several mechanisms of pathogenicity have been associated with *C. tropicalis* (Table 1.4), such as adhesion to different surfaces (Figure 1.2), biofilm formation, capacity of dissemination (Figure 1.3), hyphae and enzymes production. These factors are concisely described in Table 4. Additionally, relevant findings have been indicating higher pathogenicity for *C. tropicalis* than other NCAC species. Unfortunately, the pathogenic mechanisms of *C. tropicalis* have not been yet fully elucidated [20, 21, 66, 67, 83].

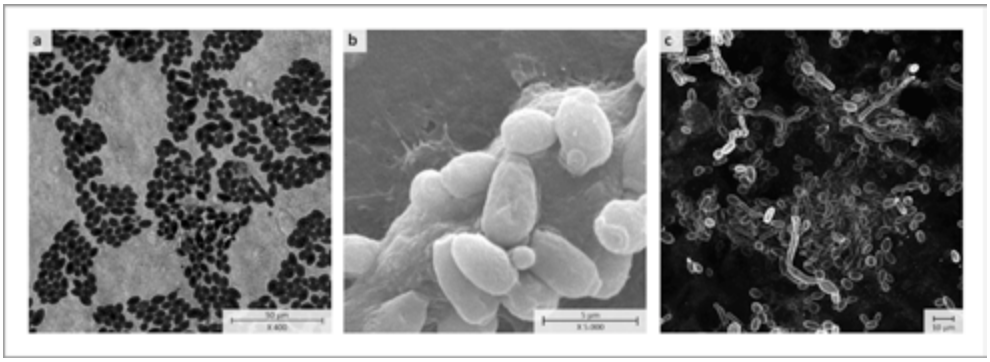

Figure 1.2: *Candida tropicalis* adhered to different surfaces: A) optical micrograph of *C. tropicalis* on silicone coupons; B) Scanning electron micrograph of *C. tropicalis* adhered to a human epithelial urinary bladder cell line; C) Confocal laser scanning microscopy image of *C. tropicalis* adhered to a reconstituted human oral epithelium.

Adhesion and biofilm formation

Candida tropicalis possesses a remarkable capacity to adhere to abiotic surfaces (Figure 1.2 a), human cells (Figure 1.2 b) and tissues (Figure 1.2 c). It is known that *Candida* cells have several different adhesins (special cell wall proteins), which allows adhesion to specific substrates. *Candida* Als (agglutinin-like sequence) is considered an important protein family during the process of adhesion, mediating attachment to different epithelium cells, functioning as an adhesion. Furthermore, southern blot analysis with *ALS*-specific probes suggested the presence of *ALS* gene families in *C. tropicalis* [84, 85].

Table 1.4: *Candida tropicalis* virulence factors analysed and major conclusions

Reference	Virulence factor	*Candida* spp. (n)	Comments
[99]	Adhesion to biotic surface and infection	*C. tropicalis* (1); *C. albicans* (1); *C. parapsilosis* (1); *C. glabrata* (1); *C. krusei* (1); *C. pseudotropicalis* (1).	*Candida* interaction with endothelium using porcine whole blood vessel. *C. albicans* and *C. tropicalis* adhered in higher extent followed by *C. krulsei*, *C. parapsilosis*, *C. pseudotropicalis*, and *C. glabrata*.
[30]	Adhesion to a biotic surface	*C. tropicalis* (2); *C. albicans* (12); *C. parapsilosis* (2); *C. glabrata* (12); *C. lusitaniae* (1); *C. kefyr* (1); *C. krusei* (2); *C. colliculosa* (1).	This work studied the adherence of different *Candida* strains isolated from the human gastrointestinal tract. Adherence to buccal epithelial cells was maximal for *C. albicans*, *C. tropicalis* and *C. parapsilosis* and minimal for *C. krusei*.
[21]	Adhesion to a biotic surface; dissemination *in vivo*; hydrophobicity; acidic conditions; sucrose assimilation.	*C. tropicalis* (5); *C. albicans* (1).	*C. tropicalis* strains were tested for their lethality in mice, adherence to Hela cells, hydrophobicity, yeast growth under acidic conditions (pH 2.0 - 5.9) and sucrose assimilation. The pathogenicity in mice by all the tested *C. tropicalis* strains was not correlated with the adherence, the hydrophobicity, or yeast growth. The pathogenicity correlated well with the sucrose assimilation ability. Pathogenic mechanisms of *C. tropicalis* strains were different from those of the *C. albicans* assayed.
[87]	Infection to epithelium; enzyme expression.	*C. tropicalis* (6).	This study investigated the infectivity of *C. tropicalis* isolates using a reconstituted human oral epithelium (RHOE) and secreted aspartyl proteinase (*SAPT*) gene expression. *SAPT1-4* genes expression was strain-dependent, with *SAPT2-4* transcripts being frequently detected and *SAPT1* rarely detected. *C. tropicalis* was highly invasive with the ability to induce significant tissue damage. These features, however, do not appear to be related to specific *SAPT* gene expression.
[86]	Adhesion and biofilm formation to abiotic surface; *Candida* surface properties.	*C. tropicalis* (2); *C. parapsilosis* (2); *C. glabrata* (2);	This work compared both the adhesion and biofilm formation on silicone of urinary clinical isolates in the presence of urine. NCAC species were able to adhere to and survive on silicone in the presence of urine. Similar water contact angle values were obtained for all NCAC strains. C. glabrata strains presented higher colonization abilities than C. tropicalis and C. parapsilosis strains.
[95]	Biofilm and drug resistance	*C. tropicalis* (1); *C. albicans* (1).	In this study, the chemical matrix composition of *C. albicans* and *C. tropicalis* biofilms and biofilm drug resistance were analysed. *C. tropicalis* biofilm matrix contained carbohydrates, proteins, hexosamine, phosphorus and uronic acid, but its major component was hexosamine, whereas in *C. albicans* matrix the major component was glucose. Biofilms of *C. tropicalis* synthesized large amounts of matrix material and such biofilms were completely resistant to both amphotericin B and fluconazole.
[23]	Biofilm and drug resistance	*C. tropicalis* (2).	This study investigated the characteristics of *C. tropicalis* biofilm development regarding the different growth phases, morphology and antifungal susceptibility. Mature biofilms consisted of a dense network of yeast cells and filamentous forms of *C. tropicalis*. Increased resistance of sessile cells against fluconazole and amphotericin B was detected. Sessile cells overexpressed ERG11 and MDR1 by real-time PCR, indicating fluconazole resistance by *C. tropicalis* biofilm.

Table 1.4 (continued.)

Reference	Virulence factor	*Candida* spp. (n)	Comments
[100]	Haemolyse activity	*C. tropicalis* (5); *C. albicans* (15); *C. dubliniensis* (2); *C. glabrata* (34); *C. parapsilosis* (5); *C. lusitaniae* (2); *C. famata* (3); *C. guilliermondii* (4); *C. rugosa* (1); *C. utilis* (1); *C. pelliculosa* (1); *C. kefyr (2)*; *C. krusei* (4); *C. zeylanoides* (1).	This is the first study demonstrating the variable expression profiles of haemolysins by different *Candida* species. Total and partial haemolysis was detectable in *C. albicans*, *C. dubliniensis*, *C. kefyr*, *C. krusei*, *C. zeylanoides*, *C. glabrata*, *C. tropicalis*, and *C. lusitaniae*. Only partial-haemolysis was detectable in *C. famata*, *C. guilliermondii*, *C. rugosa*, and *C. utilis*. No haemolytic activity was observed in *C. parapsilosis* and *C. pelliculosa*.
[101]	Ezymes production and biofilm; susceptibility testing	*C. tropicalis* (6); *C. albicans* (2); *C. glabrata* (5); *C. krusei* (3); *Candida* sp. (2).	It was evaluated the production of acid proteinase, phospholipase, haemolysin, and biofilm formation. One isolate of *C. tropicalis* had a strong positive phospholipase activity and high resistance to fluconazole. All isolates showed haemolytic activity.
[102]	Enzymes production	*C. tropicalis* (9); *C. albicans* (60); *C. glabrata* (4); *C. parapsilosis* (2); *C. lusitaniae* (1); *C. famata* (3); *C. guilliermondii* (3); *C. rugosa* (2); *C. kefyr* (4); *C. krusei* (6); *C. lipolytica* (4).	Phospholipase and proteinase activities were tested in clinical *Candida* isolates with reference to the sources of strains. Proteinase producers in NCAC species were observed as *C. kefyr*, *C. lipolytica*, *C. parapsilosis* and *C. tropicalis*. *Candida albicans* isolates tested were phospholipase producers and only a few strains of *C. glabrata* and *C. kefyr* behaved in the same way. The quantity of phospholipase produced by *C. albicans* varied with the specific isolate and correlation with the site of infection.
[103]	Enzymes production	*C. tropicalis* (19); *C. albicans* (77).	*Candida albicans* and *C. tropicalis* were obtained from whole saliva of patients presenting signs of oral candidosis. Proteinase activity was observed in both *C. albicans* and *C. tropicalis*, but phospholipase activity was only noted in *C. albicans*. *In vitro* resistance to antifungals was verified in both species, but *C. tropicalis* appears to be more resistant to the tested antifungals than *C. albicans*.
[104]	Enzymes production	*C. tropicalis* (11); *C. albicans* (24); *C. glabrata* (22); *C. krusei* (5).	The aim was to determine the enzymatic activity and to investigate the distribution of phospholipase C (PLC) gene of several *Candida* isolates from patients with pulmonary tuberculosis and non-tuberculosis patients. Phospholipase activity from *C. albicans*, *C. tropicalis* and *C. krusei* exhibited some similarity in both groups. *Candida* isolates invariably showed high levels of phospholipase, proteinase, caseinase and lipase activities.
[22]	Adhesion to abiotic surface and biofilm formation; adhesion to biotic surface; hyphae formation; enzymes production; susceptibility testing.	*C. tropicalis* (7).	Characterization of *C. tropicalis* virulence and antifungal susceptibility. All clinical isolates presented one or more virulence factors. *Candida tropicalis* strains adhered significantly more to epithelium than to silicone. All strains were able to form biofilms and to express total haemolytic activity. However, proteinase was only produced by two isolates. Only one strain was phospholipase positive. Four strains were susceptible-dose dependent to itraconazole and one clinical isolate was found to be resistant. It was not possible to establish a relation among the virulence factors assayed.

Furthermore, other factors, such as physicochemical interactions between yeast cells and materials surface, as well as environmental factors, can influence the initial adhesion of *C. tropicalis* [21, 86-88]. Several studies showed the ability of *C. tropicalis* to adhere, and consequently to form biofilms, in clinically relevant substrates like medical devices, and in different environmental situations, both *in vitro* and *in vivo.* Adherence of *Candida* cells to abiotic surfaces and to other cells is vital for biofilm formation [86, 88-91].

Candida biofilm formation is initiated when the yeast adheres to a surface, cells attach to each other and begin to proliferate, ultimately leading to the formation of a highly structured mature biofilm, comprised of complex intertwining layers of yeast, pseudohyphae and hyphae embedded in the extracellular matrix. [92, 93]. The matrix is one of the most distinctive features of a microbial biofilm. This complex extracellular material might function to defend against phagocytic cells, to serve as a scaffold for maintaining biofilm integrity, and to limit diffusion of toxic substances into the biofilm as antifungals [92, 94]. Further, studies indicate that *C. tropicalis* biofilms exhibit large amounts of matrix material completely resistant to antifungals [23, 67, 95-97]. Those data can explain why the major risk factor of *C. tropicalis* in candidosis development is related with the difficulty of treatment and, moreover, to the prolonged catheterization [53, 66, 73].

In a study done by Al-Fattani and Douglas [95], it was shown that the matrix of *C. tropicalis* contained carbohydrates, proteins, hexosamine, phosphorus and uronic acid. The major component in *C. tropicalis* matrix was hexosamine (27%), whereas in *C. albicans* matrix was glucose (32%). It is important to emphasize that hexosamine is present in *S. epidermidis* as a polysaccharide, which is sometimes referred to as the intercellular polysaccharide adhesin (PIA) and is known to mediate cell–cell interaction within the biofilm [98]. Further, in the same study, biofilms of *C. albicans* were more easily detached from plastic surfaces by treatment with the enzyme lyticase than were those of *C. tropicalis*.

However, *C. tropicalis* biofilm and consequently matrix composition are extremely dependent on environmental conditions, such as medium composition, pH, oxygen and growth conditions (static or flow) [88, 90, 96]. According to a study done by Jain *et al.* [61], comparing *Candida* biofilms grown in RPMI medium and artificial urine, biofilm formation is highly dependent on the growth medium. In particular, *C. albicans* strains produced more biofilm in artificial urine than in RPMI. Furthermore, other researches indicated that biofilms grown under conditions of continuous flow produced more matrix than those grown statically, and were significantly more resistant to amphotericin B [95].

Other important factor about biofilm life-cycle is related with dispersion/detachment or dissolution of cells, which release from the biofilm and seed new surfaces with the consequent establishment of disseminated candidosis at distal organs. Additionally, there are indications that dispersed cells from biofilms are more virulent than planktonic cells [92]. Negri et al. [90] detected that *C. tropicalis* cells are able to detach from biofilms formed in catheters under a flow of artificial urine and move upflow. However, little is still known about *C. tropicalis* detachment cells from biofilm and more studies are necessary to better understand this process.

Infection and dissemination

Adherence of *C. tropicalis* to host cells, and consequently colonization, is seen as an essential early step in the establishment of disease, since, high density colonization is indicative of high risk factor to the host [63, 66]. It is known that *C. tropicalis* is able to adhere, colonize and infect host tissues (Figure 1.3) and further disseminate, both *in vivo* and *in vitro* [21, 22, 87, 89].

It is interesting to observe that, according to some researchers, *C. tropicalis* strains showed intermediate levels of adherence to buccal epithelial cells [30, 89] and to human epithelial cell monolayers [105], whereas *C. albicans* strains showed high *in vitro* adherence. However, in others studies, *C.*

tropicalis showed similar or higher extent of adhesion than *C. albicans* when in contact with human epithelial cell monolayers [106] and endothelium from porcine vascular tissues [99]. Therefore, it is possible to verify that *Candida* species do not adhere in the same manner to the different mucosal type of cells, and also that there is distinct interaction between epithelium morphology and molecular events during *Candida* adhesion [107].

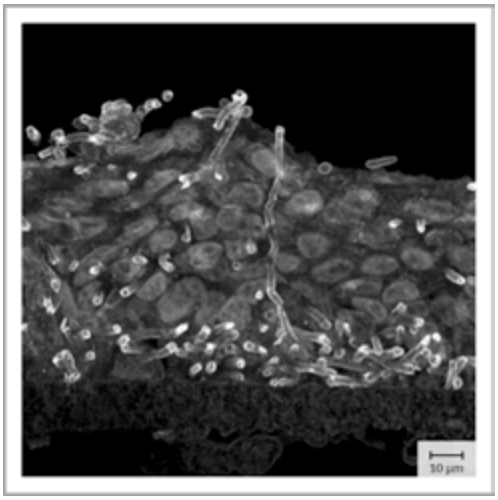

Figure 1.3: Confocal laser scanning micrograph of *C. tropicalis* infecting reconstituted human oral epithelium.

In a recent *in vivo* experimental study in mice, Okawa *et al.* [108] observed that the pathogenicity of *C. tropicalis* strains was not correlated with the adherence ability. Silva *et al.* [87] recently demonstrated that only filamentous forms of *C. tropicalis* were able to invade an oral epithelium reconstituted model. In fact, hyphae have an important role in tissue invasion, and *in vitro* research has shown that *C. albicans* lacking hyphal formation exhibited lower ability for tissue invasion compared with wild-type *C. albicans* strains [109]. The morphological forms exhibited by *C. tropicalis* are similar to those shown by *C. albicans*, but despite these few studies, there are no more evidences on the importance of *C. tropicalis* morphology in virulence. Furthermore, these studies indicate that after prolonged infection *C. tropicalis* increases its infectivity, causing more tissue damage and mice mortality [21, 87, 109]. Corroborating this fact, *C. tropicalis* was found to be highly invasive after 12 h of infection, with extensive tissue damage occurring after 24 h [87].

Thus, the pathogenic mechanisms of *C. tropicalis* seem to be different from those of *C. albicans* [21, 106]. A significant work [110] on pathogenicity of *Candida* species in an animal model, showed that the most pathogenic group was *C. albicans* and *C. tropicalis*, followed by an intermediate group with *C. glabrata*, *C. lusitaniae* and *C. kyfyr* and a least pathogenic group of *C. parapsilosis*, *C. krusei* and *C. guillerimondii*. It is important to emphasize the clinical relevance of those findings, since the major problems with the development of invasive *C. tropicalis* candidosis are associated with high dissemination and mortality.

Enzymes production

Once adhered to host cells, *C. tropicalis* requires other factors to penetrate into the deepest tissues, e.g., hyphal formation and production of enzymes. In fact, the pathogenic capabilities of this yeast have been related to the secretion of aspartyl proteinases, phospholipases and haemolytic activity [22, 24, 25, 67, 85, 111].

Candida produce a large variety of secreted hydrolases, and among the various potential virulence factors proposed, the secreted aspartyl proteinases (Sap) have been intensively investigated. It is now well established that the ability of *C. albicans* to adhere to mucosae, to invade in deep organs, and to resist to phagocytic cells, apparently requires the use of several different proteinases suitable to each particular condition during the infection. Like *C. albicans, C. tropicalis* presents *in vitro* Sap activity in a medium containing bovine serum albumin (BSA) as the sole source of nitrogen [22, 31, 85, 112].

Furthermore, Zaugg *et al.* [24] characterized a total of four *SAPT* genes family of *C. tropicalis*. According to this study, RT-PCR experiments revealed a strong *SAPT1* signal with RNA extracted from cells grown in BSA medium. The *SAPT2* and *SAPT3* gene products, Sapt2p and Sapt3p, which have not yet been detected in *C. tropicalis* cultures *in vitro*, were produced as active recombinant enzymes with the methylotrophic yeast *Pichia pastoris* as an expression system. However, a weak signal was obtained with all other *SAPT* genes under *in vitro* conditions tested, suggesting that

the gene products Sapt2p, Sapt3p, and Sapt4p could be produced during infection.

This idea is highlighted by recent studies [87, 109] that investigated epithelial infection by *C. tropicalis* using a reconstituted human oral epithelium (RHOE) and *SAPT* gene expression. The results obtained by real-time PCR, showed that *C. tropicalis* isolates were able to express *SAPT1*-4 during the infection process. Moreover, expression was strain-dependent, with *SAPT2-4* transcripts being frequently detected and *SAPT1* rarely detected. Furthermore, *C. tropicalis* can be considered as highly invasive with the ability to induce significant tissue damage [87]. These features, however, do not appear to be related to specific *SAPT* gene expression. Therefore it is necessary to perform more investigations focusing on *SAPT* genes family of *C. tropicalis* for better understanding the specific role of these genes.

In addition to Saps, enzymes categorized as lipases (LIPs), this means enzymes that hydrolyze phospholipids into fatty acids, are often considered to be involved in *C. tropicalis* pathogenicity and are suggested to contribute to host cell membrane damage which could also expose receptors to facilitate adherence [102, 111, 113]. In *C. albicans*, 10 genes encoding for LIPs (*LIP1-10*) have been identified and similar sequences were also detected in *C. tropicalis* [114]. Nevertheless, the most widely used diagnostic method for phospholipases (PLs) determination is based on yeast growth in an egg yolk agar medium [115]. According to recent studies, using this method, *C. tropicalis,* from different sources, appears to have a reduced ability to produce extracellular PLs *in vitro* when compared with *C. albicans* [29, 102, 103]. However, this production is highly species and strain dependent [22, 25, 101, 116].

Other important virulence factor recently described in literature is related with haemolytic activity which is tested on sheep blood agar supplemented with glucose [100]. It is known that enzymes as haemolysins are used by *Candida* species to degrade haemoglobin and facilitate recovery of the elemental iron from host cells, which is a contribute to pathogenicity in

Candida species. Thus, haemolysins are considered key virulence factors enabling pathogen survival and persistence in the host [100, 117, 118]. The studies reported so far show that *C. tropicalis* are all able to produce haemolysins *in vitro*, inducing partial or total erythrocyte lyses, although the degree being strain dependent [100]. According to Luo *et al*. [100], total-haemolytic activities in *C. albicans* and *C. tropicalis* were significantly higher than in *C. glabrata*. However, Kumar et al. [104] observed the opposite, *C. glabrata* displayed the highest haemolytic activity when compared with *C. albicans* and *C. tropicalis*. Despite significant studies showed the ability of *C. tropicalis* to produce haemolytic activity on sheep blood agar supplemented with glucose, it is important to assess whether the haemolytic activity observed is true or is a product of extracellular PLs of *Candida* species. Moreover, it is still necessary to have more advances in molecular studies to clarify the role of haemolytic activity in *C. tropicalis* pathogenesis.

CONCLUDING REMARKS

In fact, the frequency of *Candida tropicalis* causing candidosis is increasing in the last decades, probably due to several situations, e.g., new and efficient molecular methods of identification; antifungal resistance mainly to fluconazole commonly used as prophylaxis agent; and factors related with host as well as invasiveness surgery, long time in ICU, antibiotic administration and catheterization. Additionally, invasive disease developed by *C. tropicalis* is associated with colonization, high potential of dissemination and pathogenicity by this organism. Mainly because *C. tropicalis* possesses a diversity of virulence factors that induces serious damage to patients and increases the mortality risk. However, it is necessary much more research to get deeper insights into the strategies used by *C. tropicalis* to change from a harmless commensal microorganism to become a human pathogen of high clinical concern.

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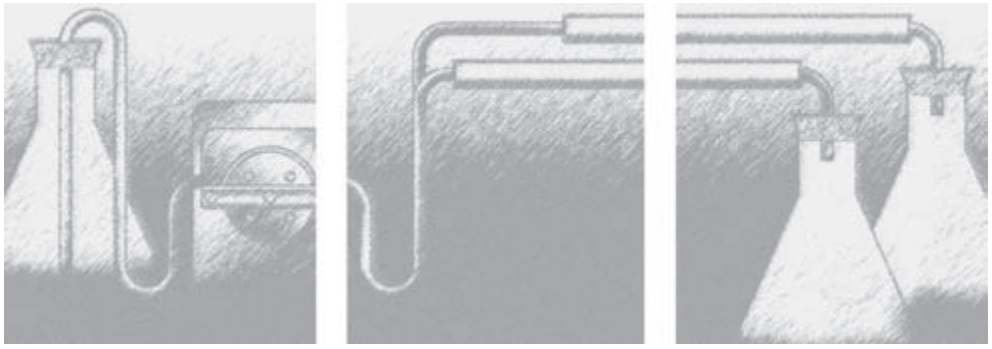
CHAPTER 2

Candida tropicalis biofilms: artificial urine, urinary catheters and flow model



ABSTRACT

Adhesion to medical devices and biofilm formation are considered important virulence factors of *Candida tropicalis*. This work aimed to use artificial urine (AU) and urinary catheters, under flow conditions, for studying *C. tropicalis* biofilms. Adhesion and biofilm formation on silicone and latex urinary catheters were quantified by crystal violet staining and determination of colony forming units. *Candida* surface hydrophobicity was also evaluated, as well as the biofilms' matrix content in terms of proteins and carbohydrates. *Candida tropicalis* was able to adhere and to form biofilms along the entire length of the catheters under flow conditions. It was found that the isolate U69 adhered significantly more to both types of catheters than did the reference strain. However, U69 biofilms contained significantly less cultivable cells and higher biofilm biomass than those of the reference strain. Detachment of cells from biofilms on latex catheter was lower compared to silicone catheter. This model using AU appeared to be suitable for studies mimicking the real body conditions. Additionally, *C. tropicalis* was in fact able to colonize urinary catheters in the presence of AU and to detach from these catheters, demonstrating their capacity to colonize distal sites.

Keywords: *Candida tropicalis*, urinary catheter, flow conditions, biofilm, cell detachment

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INTRODUCTION

Urinary tract infections (UTI) are usually the most common type found in hospitals in developed countries. Although medical devices are indispensable in the management of critically ill patients, about 20% of fungal UTI are associated with the use of urinary catheters [1-3]. *Candida* species are the most frequently isolated fungi, corresponding to approximately 80% of fungal associated nosocomial infections [1, 4, 5] and are the second most common species responsible for patient mortality [6, 7]. Moreover, candiduria has increased in recent years among patients admitted to intensive care units (ICUs), especially those requiring prolonged urinary catheterization or receiving broad-spectrum antibiotics [6, 8].

Candida tropicalis is usually considered to be the first or the second most common non- *Candida albicans Candida* (NCAC) species isolated from the urinary tract [6, 8]. Additionally, *C. tropicalis* is often recovered from patients admitted in ICUs, particularly those with cancer, requiring prolonged catheterization, or receiving broad-spectrum antibiotics [9, 10]. Several virulence factors seem to be responsible for *C*. *tropicalis* infections, which present high potential for dissemination and mortality [11, 12]. Adhesion to medical devices as well as biofilm formation are considered important factors in these infection [13-15] and this yeast is able to form extensive biofilms *in vitro* on the surface of catheters [16-18].

Various model systems have been used to characterize the ability of *Candida* species to form biofilms but these procedures usually consider biofilm formation under static conditions [19-22]. However, biofilm formation *in vivo* is often subjected to a liquid flow and to both physical and environmental factors of the human host [19, 23]. Furthermore, shear force and replenishing nutrients play a key role in biofilm development and are known to alter biofilm growth and architecture [13, 19-21].

Moreover, different studies have reported that *Candida* biofilm formation under flow model conditions show better correlation with biofilm

formation *in vivo* than do static models [18-20, 24]. Although there have been some studies using flow models [18-20, 24, 25], the information on *C. tropicalis* behaviour under medically relevant situations is still limited. Thus, the principal aim of this work was to test a model to evaluate the extent of the influence of dynamic conditions on *C. tropicalis in vitro* biofilm formation on urinary catheters (silicone and latex) using artificial urine.

MATERIAL AND METHODS

Organisms and growth conditions

In addition to the reference *C. tropicalis* strain (ATCC 750), this study was conducted with one isolate of *C. tropicalis* (U69) obtained from the collection of the University Hospital in Maringá, Paraná, Brazil. It had been initially recovered from a patient with candiduria who had been admitted to the intensive care unit of the hospital.

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h at 37 °C. Cells were then inoculated into Sabouraud dextrose broth (SDB; Merck) and incubated for 18 h at 37 °C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 8000 g for 5 min at 4 °C and washed twice with phosphate buffer solution (PBS). The remaining pellets were suspended in artificial urine (AU) and the cellular density adjusted to 1 x 10^5 cells/ml using a Neubauer chamber. Artificial urine (pH 5.8) was prepared according to Silva *et al*. [13], with $CaCl_2$ (0.65 g/l), $MgCl_2$ (0.65 g/l), NaCl (4.6 g/l), Na_2SO_4 (2.3 g/l), $Na_3C_3H_5O(CO_2)_3$ (0.65 g/l), $Na_2C_2O_4$ (0.02 g/l), KH_2PO_4 (2.8 g/l), KCl (1.6 g/l), NH_4Cl (1.0 g/l), urea (25.0 g/l), creatinine (1.1 g/l), and dextrose (0.3%).

Candida tropicalis biofilm formation flow model

Biofilms were developed on indwelling urinary catheters under simple continuous flow. Two different commercially available urinary catheters of the same size (5.3 mm external diameter and 250 mm length), and in common use in Portuguese clinical practice, were employed in the studies, i.e., silicone, (Silkemed Uro-Cath Balloon, Algália de Foley 100% silicone, Overpharma, Portugal) and latex (Silkemed Uro-Cath Balloon, Algália de Foley em latex, Overpharma, Portugal). To investigate the formation of biofilms, the ends of the catheters were first cut aseptically (final total catheter length 200 mm) and a system of continuous flow was constructed connecting the remaining catheter to a feeding flask containing the yeast suspension in AU using a sterile silicone tube (Figure 2.1). The flow in each catheter was controlled (1 ml/min) with a peristaltic pump (Reglo Analog MS-2/6, Iswatec, Labortechnik – Analytik, Switzerland).

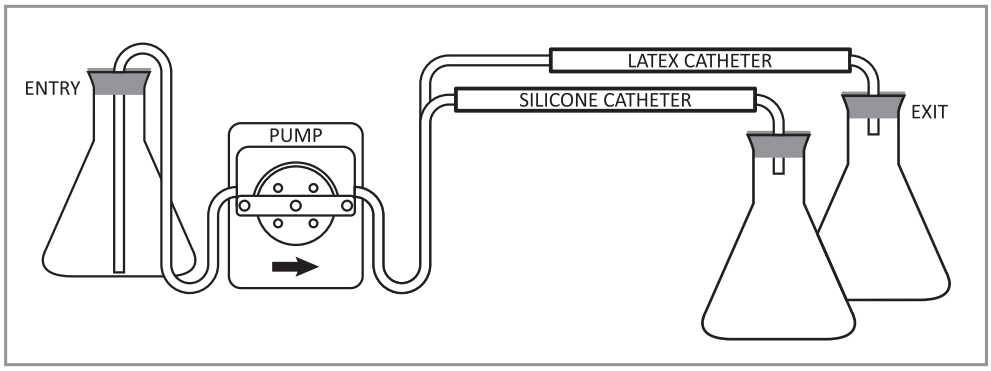

Figure 2.1: Schematic representation of the catheter flow model, including a peristaltic pump, waste flasks, inoculation flask, and common catheters.

Cells were then passed through each catheter for 2 h and the entire system was kept in an incubator at 37 °C. After this preliminary step of cell adhesion, the flask containing the yeast suspension was replaced by another flask containing only sterile AU, the medium flow was set to a constant rate of 1 ml/min and the entire system was again maintained at 37°C.

After cell adhesion (2 h) and biofilms formation (24 h), the catheters were removed aseptically to evaluate the concentration of cultivable yeast, biofilm biomass, biofilm matrix composition and to conduct scanning

electron microscope studies. All the experiments were performed in triplicate and in three independent assays.

Candida cells quantification

Adhesion or biofilm samples

The culture medium was removed from the catheter by passing air through the flow system and adhered or biofilm cells were scraped into PBS, after which each catheter was cut in small portions (10 mm). The time and potency of sonication had been previously established to optimize the complete removal of the adhered cells without causing any damage to them (optimization was followed with crystal violet staining and the determination of CFU). The catheter portions immersed in PBS were sonicated (Ultrasonic Processor, Cole- Parmer, Illinois, USA) for 45 s at 30 W or 50 s at 30 W for the silicone and the latex catheters, respectively. Then the resultant suspension was vortexed for 2 min and serial dilutions in PBS were inoculated onto SDA plates and incubated for 24 h at 37 °C. The total colony forming units (CFUs) were counted per unit area (CFU/cm^2) of catheter. These catheter portions were stained using the crystal violet (CV) method [14, 21] to verify the complete removal of the cells.

Biofilm detached cells

After the first 2 h of adhesion, the cells released from the catheters were collected (1 ml) at various time points during biofilm development at both the entry and exit of the flow system. The dispersed cells were determined by enumerating the CFUs.

Biofilm biomass

Biofilm formation was assessed by total biomass quantification using CV staining [14, 21]. After biofilm formation (24 h), the catheters were cut as described previously into sections. The catheter portions containing

biofilms were transferred to 24-wells polystyrene microtiter plates (Orange Scientific c, Braine-l' Alleud, Belgium) and fixed for 15 min with 1 ml of 100% (v/v) methanol. The catheter portions were then allowed to dry at room temperature, and 1 ml of CV (1% v/v) was added to each well and incubated for 5 min. The samples were gently washed with sterile water and 1 ml of acetic acid (33% v/v) was added to release the CV from the biofilm. The absorbance of the resultant solution was read in triplicate in a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at λ=570 nm. The final absorbance was standardized according to the volume of acetic acid and area of the catheter (absorbance/cm^2). The *Candida* biofilms' quantification was performed in triplicate and in three independent assays.

Scanning electron microscopy (SEM)

Catheter portions were prepared as described in biofilm biomass quantification and they were transferred to 24-well polystyrene microtiter plates. The samples were dehydrated with alcohol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until each catheter portion was removed for analysis. Prior to observation, the catheter portions were mounted onto aluminium stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, USA).

Biofilm matrix composition

Extraction method

The biofilm matrix was extracted in accord with the procedures described by Silva *et al*. [26]. Briefly, biofilm samples were prepared as described before, sonicated and vortexed. Then the suspension was centrifuged at 3000 g for 10 min at 4 °C and the supernatant (containing the matrix) filtered through a 0.2 mm nitrocellulose filter and stored at -20 °C prior to analysis. The pellets were dried at 60 °C to obtain a constant dry biofilm weight.

Protein and carbohydrate quantification

The protein content of the biofilm matrix was measured using the BCA Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, MO, USA), with bovine serum albumin (BSA) as standard. Total carbohydrate content of the biofilm matrix was estimated according to the procedure of Dubois *et al.* [27], employing glucose as the standard. The biofilm matrix composition was determined in triplicate and in three independent assays.

Candida surface properties

Contact angle measurement

The degree of hydrophobicity (ΔG_{sws}) was evaluated through contact angle measurements using the approach of van Oss *et al*. [28]. The measurements were made on *Candida* cell lawns on membrane filters, prepared according to Busscher *et al*. [29]. For this, suspensions of 5 ml of 1 x 10^8 cells/ml in AU were filtered, inoculated over solidified agar plates (2% agar and 10% glycerol) and dried at 37 °C for 3 – 4 h to standardize the humidity level. Contact angles were measured by the sessile drop technique on the cell lawns, using a contact angle measurement apparatus (model OCA 15 Plus, Dataphysics). In order to determine the degree of hydrophobicity (ΔG_{sws}), the measurements were made at room temperature, using three different liquids, i.e., water, formamide and 1-bromonaphtalene. Water contact angles were used as a qualitative indication of the cell surface hydrophobicity, with an angle lower than 65° indicating a more hydrophilic surface [30]. Each assay was performed in triplicate and at least 20 contact angles, per sample, were measured.

Statistical analysis

Results obtained were analysed using the SPSS 18 version (Statistical Package for the Social Sciences) program. Onaway ANOVA with the Bonferroni test was used to compare the number of adherent or biofilm cells and matrix composition of the strains assayed. All tests were performed with a confidence level of 95%. All the experiments were performed in triplicate and in three independent assays.

RESULTS

Candida tropicalis adhesion and surface properties

We found (Table 2.1) that isolate U69 adhered significantly ($P < 0.05$) more to silicone than to latex catheters and with respect to the silicone catheter it adhered (3.78 x 10^3 CFU/cm^2) to a greater extent than the reference strain (1.49 x 10^3 CFU/cm^2). However, the opposite was found with the latex catheters. SEM images (Figure 2.2) clearly show the differences between latex and silicone catheter materials, with latex (Figure 2.2; i) catheters having more irregular surfaces than silicone (Figure 2.2; ii).

Table 2.1: Number of *C. tropicalis* cells adhered (2h) to both types of catheters, water contact angle (θ), surface tension parameters (γ^+, γ^-), and degree of hydrophobicity (ΔG_{sws}) of planktonic cells. The values are means ± standard deviations from three independent experiments for each condition

Strain	Adhesion (CFU/cm^2x10^3)		Surface parameters			
	Latex	Silicone	θ (°)	γ^+ (mJm^2)	γ^- (mJm^2)	ΔG_{sws} (mJm^2)
Isolate U69	*1.04 ± 0.23	*3.78 ± 1.19	30.4 ± 1.6	0.1	57.0	43.8
Reference strain	1.76 ± 0.02	1.49 ± 0.39	20.5 ± 2.3	4.5	49.8	23.0

* Statistical differences ($P < 0.05$) between materials and in each strain.

Table 2.1 also shows *Candida* surface physic-chemical characteristics, i.e., the degree of hydrophobicity (ΔG_{sws}). The water contact angles obtained for both *C. tropicalis* test isolates are low indicating hydrophilic surfaces. Moreover, the ΔG_{sws} values also show that both strains are hydrophilic (ΔG_{sws}), with isolate U69 displaying a greater hydrophilic character. As to surface tension components, *C. tropicalis* had higher electron donor parameter (γ^-) values compared to the electron acceptor parameter (γ^+), and the reference strain showed a higher value of electron acceptance (4.5 mJm^2) than isolate U69 (0.1 mJm^2).

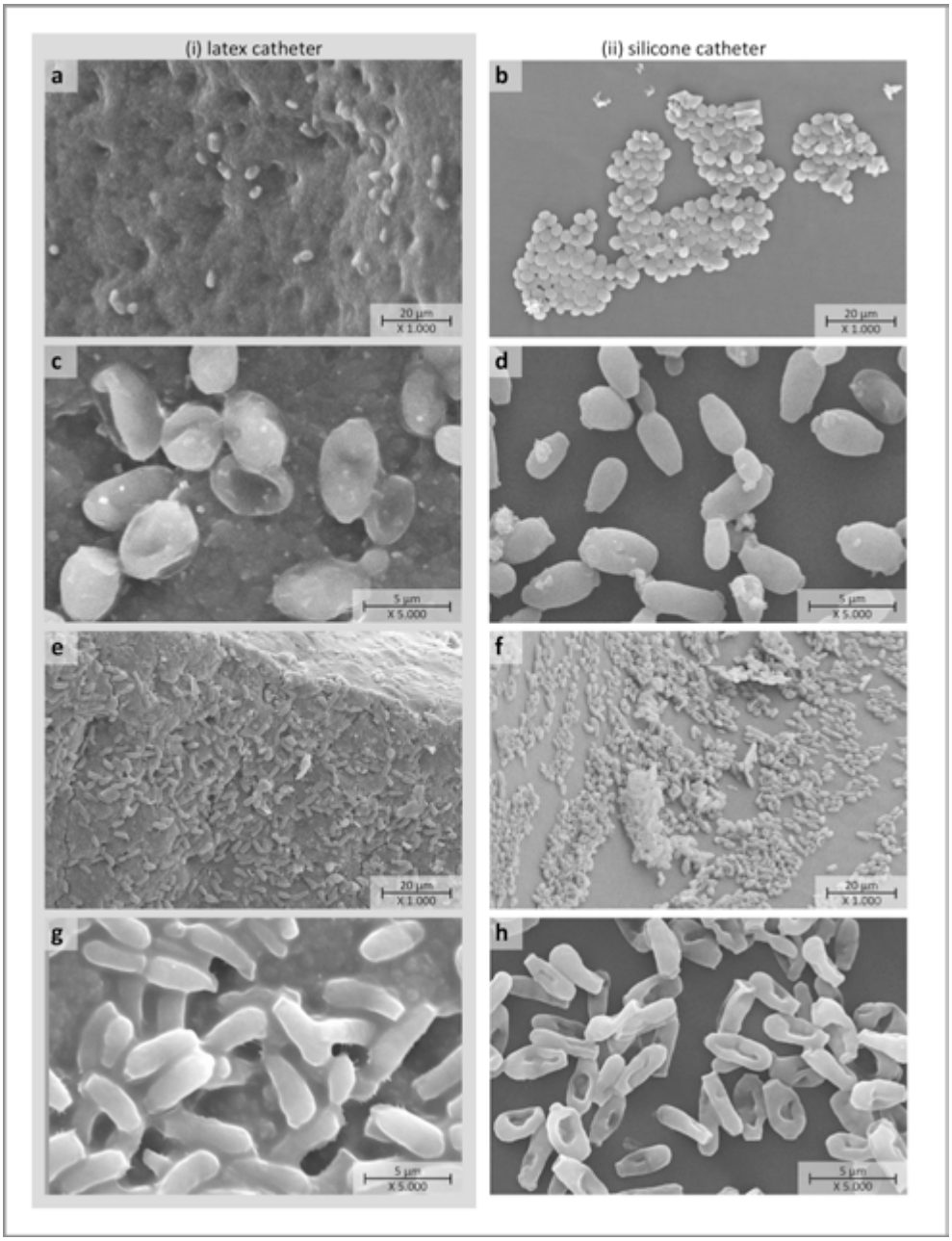

Figure 2.2: SEM images of initial adhesion (a–d) and biofilm formation (e–h) on latex (i) and silicone (ii) catheters by *Candida tropicalis* isolate U69.

Biofilm development under flow conditions

It was possible to observe that *C. tropicalis* was able to form biofilm along the entire length of the urinary catheter in the presence of AU (Figure 2.3).

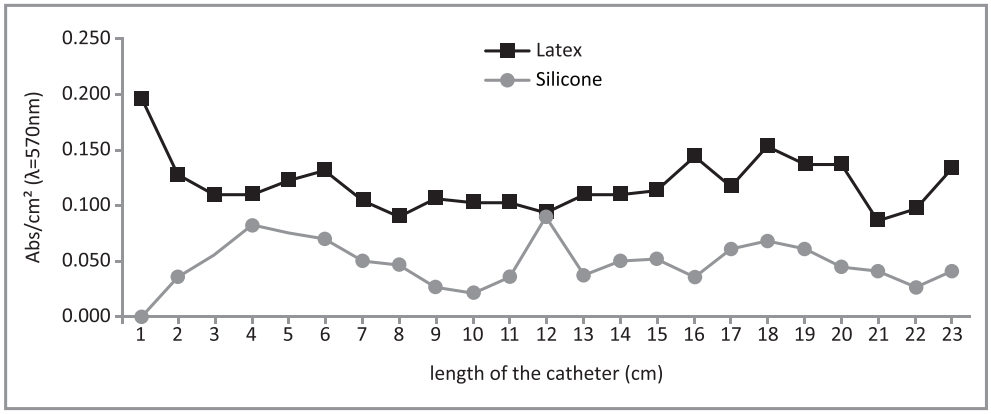

Figure 2.3: *Candida tropicalis* reference strain (ATCC 750) biofilm (24 h) along the length of the silicone and latex urinary catheters, analysed by CV staining method.

It was possible to observe an increase in the number of cells of both strains (Tables 2.1 and 2.2, and Figure 2.2) for from 2 – 24 h but there were no significant differences after 24 h (data not shown). Moreover, *C. tropicalis* isolate U69 biofilm (Table 2.2) contained significantly ($P < 0.005$) less CFUs than the reference strain on both silicone and latex catheters. However, it presented a higher biofilm biomass especially on latex catheter (Abs/cm^2 = 0.123 and 0.079 for isolate U69 and reference strain, respectively). The yield of total proteins and carbohydrates extracted from the matrix of biofilms formed on the two types of catheters are presented in Table 2.2. In general, biofilm matrices composed of both *C. tropicalis* strains had high amounts of proteins and relatively low amounts of carbohydrates. Moreover, the matrix of biofilms formed on the latex catheter had statistically higher protein content when compared to the ones formed on the silicone catheter. Interestingly, biofilm matrices of isolate U69 had relatively higher quantities of both protein and carbohydrate compared to the matrix of the reference strain biofilm.

Table 2.2: Number of cultivable cells after 24 h, biofilm biomass expressed as CV absorbance and biofilm matrix composition (protein and carbohydrate) of both *C. tropicalis* isolates on silicone and latex catheters obtained from biofilms formed in artificial urine. The values are means ± standard deviations from three independent experiments for each condition

Strain			Isolate U69	Reference strain
Cultivable cells enumeration (CFU/cm^2x10^3)		Latex	*1.39 ± 0.56	26.7 ± 3.51
		Silicone	*7.08 ± 2.20	17.7 ± 2.0
Biofilm biomass (Abs/cm^2)		Latex	*0.123 ± 0.026	0.079 ± 0.017
		Silicone	0.048 ± 0.020	*0.033 ± 0.010
Matrix component (mg/g of biofilm dry weight)	Protein	Latex	*†53.6 ± 5.6	*†6.6 ± 0.8
		Silicone	*15.8 ± 4.6	*0.9 ± 0.5
	Carbohydrate	Latex	0.02 ± 0.00	0.01 ± 0.00
		Silicone	0.03 ± 0.01	0.001 ± 0.00

* Statistical differences between strains ($P < 0.05$)
† Statistical differences between materials ($P < 0.05$)

Biofilm cells detachment

The detachment of cells from isolate U69 biofilms (Figure 2.4) on latex catheter was lower when compared with the results of dispersed cells recovered from the silicone catheter. Furthermore, when biofilms were formed on silicone it could be observed that a high number of cells were dispersed at 24 h. Regarding the cells recovered in the inlet, we found that some cells (3.60×10^2 CFU/ml for latex; 1.56×10^2 CFU/ml for silicone) detached from biofilms and followed the reverse way (back to the feed flask). This chemotaxis phenomenon was only found after 96 h of continuous flow.

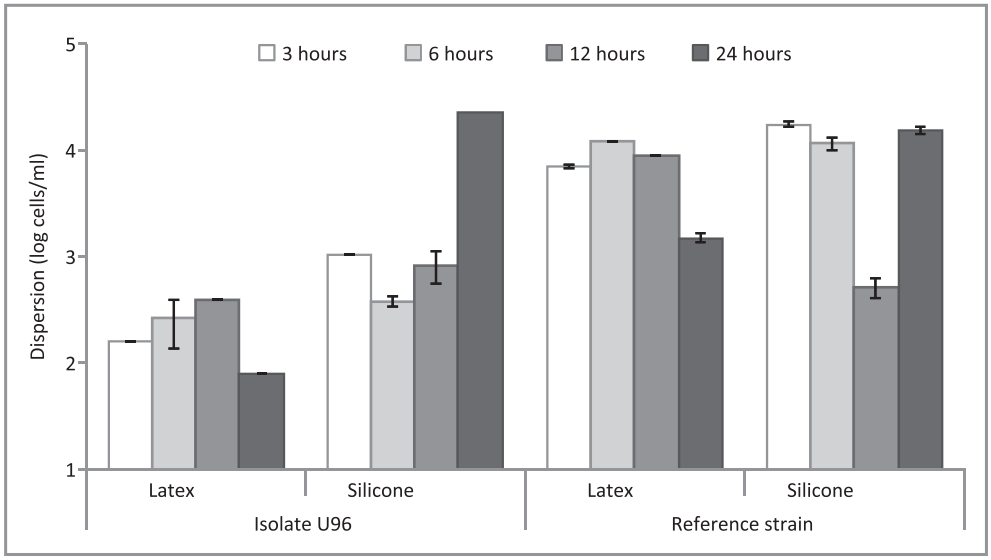

Figure 2.4: Detachment of cells from *Candida tropicalis* biofilms grown in artificial urine. The number of dispersed cells recovered from *C. tropicalis* biofilms was quantified during 3, 6, 12 and 24 h. Results shown are expressed as mean and standard deviation from three independent experiments for each condition.

DISCUSSION

Candida tropicalis has been considered the species most frequently isolated from *Candida* infections in the Pacific-Asia region [31], Brazil [32, 33], and recently in Europe [5, 34]. In the majority of these cases, infections were associated with biofilm formation on medical devices used with patients admitted in intensive care units (ICUs) [9, 10, 33].

Although there are some studies that used *in vitro* flow models to attempt to mimic *Candida* biofilm development *in vivo* [16, 17, 20, 24, 35, 36], to the authors' knowledge, the information on the behaviour of *Candida* species under human body conditions is still limited. Generally, studies regarding *C. tropicalis* biofilms have used static models [13-15, 26] employing only silicone coupons or discs of catheter material and rich media, which are quite dissimilar from actual clinical conditions. Thus, the system developed in this work using AU overcomes these drawbacks and in addition, proved to be simple and easily reproducible in any laboratory.

Furthermore, this model could readily be applied with all kinds of catheters (urinary, central venous, or parentheric), body fluids, using the representative flow, and microorganisms that mimic host physical and environmental factors. Moreover, it was possible to use two different types of catheters simultaneously and thereby, increasing the reliability of the results.

Concerning the adhesion process, biomaterial surface roughness is a relevant property for *Candida* attachment, with the irregularities of the polymeric surfaces normally promoting *Candida* adhesion and consequently biofilm accumulation [26, 37, 38]. By examining SEM images (Figure 2.2) it is possible to detect differences between catheter materials, with latex catheters having more irregular surfaces than silicone catheters. In addition Sousa *et al*. [38], recently demonstrated that silicone exhibits large numbers of depressions and grooves. However, we observed (Table 2.1) that isolate U69 adhered to a significantly higher extent to the silicone catheters than to the latex ones (P = 0.001) and the number of adhered cells was similar for the reference strain on both catheters. In addition, biofilm formation was not directly related to roughness. Thus, it is not possible to establish a direct relationship between catheter morphology and the extent of adhesion of *C. tropicalis*.

Furthermore, other factors, such as surface properties of both microorganisms and catheter materials, as well as environmental factors [19, 35], can influence the initial adhesion of *Candida* species under continuous flow conditions. During the adhesion process, microorganisms have adhered firmly to the biomaterial surface through physicochemical interactions [38, 39]. In biological systems, hydrophobic interactions are normally the strongest of the long-range non-covalent interactions and can be defined as the attraction among a polar or slightly polar cells or molecules themselves, when immersed in an aqueous medium [28, 38]. *Candida tropicalis* surface physicochemical characteristics were evaluated through contact angles measurement, surface tension parameters, and degree of hydrophobicity.

The water contact angles obtained for *C. tropicalis* are low and the ΔG_{sws} was positive, both of which are in agreement and indicate surface hydrophilic character of *Candida* cells (Table 2.1). Moreover, according to Kazmierska *et al*. [40] silicone appears to be more hydrophobic than latex and according to Sousa *et al*. [38] the higher surface hydrophobicity of silicone is probably responsible for the highest levels of initial cell adhesion to this substrate. However, isolate U69, which is the most hydrophilic strain (higher ΔG_{sws}), presented the highest extent of adhesion to silicone, the most hydrophobic material (Table 2.1). Therefore, based on these results (Table 2.1) no direct correlation was found between adhesion capability and the degree of cell hydrophobicity. This is in accord with other reports [13, 37], and demonstrates that cell hydrophobicity alone cannot be used for predicting the microbial adhesion. Another factor that is usually involved in determining the extent of adhesion is the surface tension components of the microorganism [38]. Cell surfaces of *C. tropicalis* strains were found (Table 2.1) to be predominantly electron donors (higher values of γ^-). This polar character can be due to the presence of residual water of hydration or polar groups [28]. However, the low value of the electron acceptor parameter ($\gamma^+ = 0.1$) of isolate U69 can also justify its highest adhesion to the more hydrophobic material such as silicone.

The extent of adhesion of *C. tropicalis* to these urinary catheters is in accordance to Silva *et al*. [13], who described the adherence of NCAC species, such as *C. tropicalis*, on silicone in the presence of artificial urine. After the initial process of attachment of individual cells to a substratum, what follows is the proliferation and formation of a highly structured mature biofilm comprised of complex intertwining layers of yeast, pseudohyphae and hyphae embedded in extracellular matrix [19, 24]. *Candida tropicalis* was able to form biofilms along the entire length of urinary catheters in the presence of AU (Figure 2.3). Furthermore SEM images revealed an increase in the number of cells attached to both types of catheters from 2 h (Figure 2.2 a – d) to 24 h (Figure 2.2 e – h). In fact, *Candida* species are able to adhere and develop biofilms in the presence of AU with the number of cells proliferating from the adhesion stage to

biofilm formation [13, 41]. These data corroborate the *in vivo* situation when *Candida* biofilms grow on urinary catheters, which are intermittently bathed by patients' urine as the only source of nutrients, and are undoubtedly responsible for patients' infections. However, in this work, there was no direct correlation between the number of adhered cells and the number of biofilm cells. In fact the strain displaying the highest CFU number in initial adhesion (isolate U69) presented less CFUs in biofilms. Nevertheless, other authors [13, 14, 35, 37] have reported a correlation between the extent of adhesion and biofilm formation. However, Cerca *et al*. [42] have demonstrated that initial adhesion and biofilm formation are not always directly related and that this fact can be determined by cell-to-cell adhesion ability to make possible more than one cell layer.

Regarding biofilms, one of their most important characteristics is the presence and the composition of the extracellular matrix [17, 19], which can act as a barrier to diffusion of antimicrobial agents, thereby limiting access of antimicrobials to organisms at the base of the biofilm [17, 43]. Al-Fattani and Douglas [17], chemically analysed the matrix material from *C. tropicalis* biofilms and reported the synthesis of large concentrations of extrapolymeric material composed of hexosamine, carbohydrates, proteins, phosphorus, and uronic acid. In this study biofilm biomass and matrix composition (proteins and carbohydrates) was also evaluated and a higher amount of biofilm biomass and matrix proteins content were verified for biofilms formed on latex catheter than on silicone (Table 2.2).

Formation of mature biofilms and consequent production of matrix is strongly dependent on environmental conditions, such as medium composition, pH and oxygen [13, 26, 41]. Furthermore, some studies [35, 43-45] indicated that specific proteins are associated to adhesion, biofilm formation and dispersal of cells and the expressed quantities of these proteins depend not only on the microorganism but also on the surface where the biofilm is formed. In addition, biofilm matrices of isolate U69 had relatively higher quantities of both proteins and carbohydrates, and presented smaller CFU values compared to the reference strain.

Formation of biofilms allows microbial pathogens to create a safe niche in which sessile cells remain in a protected environment. However, cells within a biofilm may be also confronted with adverse environmental conditions (i.e., reduced nutrient availability, accumulation of toxic waste products) so the dispersion of cells is also beneficial for their survival. Furthermore, this release of cells from the original biofilm community is required to generate new communities at other locations and the dispersal of cells from biofilms may be a critical step in biomaterial related cases of candidosis [35, 36, 43]. In this work, it was curious to note that cells detached from the biofilms started to follow backwards after 96 h (data not shown), confirming the possibility of colonization of distant host sites. Still it was possible to observe that cells were dispersed from biofilms from the first time points (Figure 2.4) and the number of dispersed cells recovered from latex catheter was lower than cells recovered from silicone catheter. The highest cell detachment from silicone catheter at 24 h was noted with U69. Accordingly, Uppuluri *et al*. [36] reported that dispersion occurs at all stages of the biofilm developmental cycle and is influenced by nutritional and other physiochemical conditions.

In conclusion, the model presented allows the easy study of *Candida* biofilm lifecycle and can better mimic the host physical and environmental conditions than the traditional systems. It was also possible to conclude that *C. tropicalis* were able to form biofilms in artificial urine on different urinary catheters under flow condition. In all situations there was a significant production of matrix components. As expected, cells were released constantly from biofilms, and were recovered at the inlet after 96 h, highlighting their great effect as human pathogens. However, there was no direct relation between cells and material surface properties (degree of hydrophobicity, surface tension and materials surface morphology) and adhesion capability and biofilm formation.

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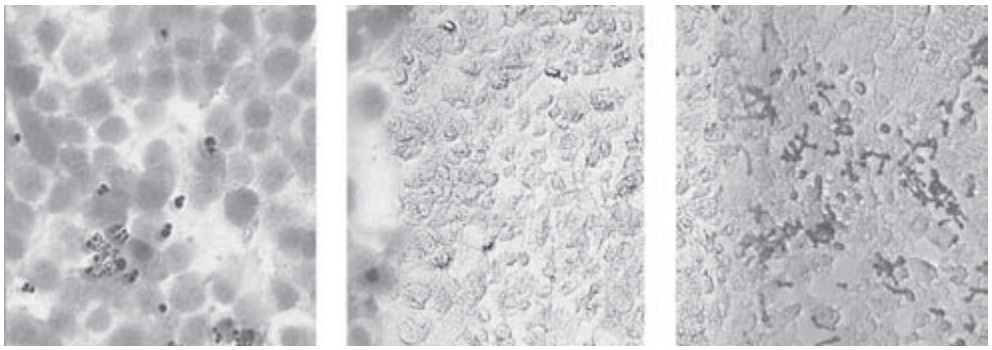
CHAPTER 3

Crystal violet staining to quantify *Candida* adhesion to epithelial cells



ABSTRACT

In vitro studies of adhesion capability are essential to characterise the virulence of *Candida* species. However, the assessment of adhesion by traditional methods is time consuming. The aim of the present study is the development of a simple methodology using crystal violet staining to quantify *in vitro* adhesion of different *Candida* species to epithelial cells. The experiments are performed using *Candida albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750). A human urinary bladder epithelial cell line (TCC-SUP) is used. Yeast and epithelial cells were stained with crystal violet, epithelial cells were then distained using intermediate washing, and the dye in the yeast cells was extracted with acetic acid. The method was validated for the different *Candida* reference species by comparison with traditional microscope observation and enumeration. The method was then used to assess *Candida* adhesion to epithelial cells and also to silicone. For all *Candida* species High correlation values (r^2= 0.9724 – 0.9997) between the number of adherent yeasts (microscope enumeration) and absorbance values were obtained for an inoculum concentration > 10^6 cells/ml. The proposed technique was easy to perform and reproducible, enabling the determination of adhesion ability of *Candida* species to an epithelial cell line.

Keywords: Adhesion; *Candida*; cell line; epithelial cells; gentian violet; silicone.

Negri M, Gonçalves V, Silva S*,* Henriques M, Azeredo J, Oliveira R. Crystal violet expedite method for quantification adhesion to epithelial cells. British Journal of Biomedical Science*,* 2010; 67 (3): 120-125.

INTRODUCTION

Candida species can adhere to a variety of different surfaces in the human body, thus facilitating the colonisation of many host niches. Remarkably, such niches provide very different environments for growth, and *Candida* has developed specific mechanisms to adapt to the respective conditions. Several studies have shown a correlation between adhesion of organisms and their potential virulence [1-4].

Adhesion to epithelial cells is well recognised as an essential step in the process of *Candida* colonisation and subsequent infection [5]. *Candida* adhesion to epithelial cells has been investigated to define parameters relevant to the pathogenesis of oral, gastrointestinal, vaginal and urinary candidiasis [6]. Furthermore, *Candida* can also grow on abiotic surfaces (e.g., plastic devices), for example, leading to biofilm formation in catheters, which represents a major problem especially in intensive care units [2, 7].

Over the past decades, a broad range of model systems have been described for the *in vitro* study of *Candida* adhesion to hard surfaces [8]. In most model systems, quantification of yeast cells is obtained by plating, which is labour-intensive and slow [9]. Moreover, yeast adhesion to epithelium can be determined by visual methods (e.g., light, fluorescence, scanning or transmission electron microscopy) or by counting radiolabelled yeast [6].

The visual method involves incubating standard yeast suspensions with confluent cell monolayers grown on a coverslip. Following removal of unattached yeasts, the number of adherent organisms per unit area of the monolayer is determined by direct microscopy after air drying, Gram staining and mounting on glass slides. Although visualisation allows monitoring of adhesion to individual epithelial cells, it is a very time-consuming technique [10, 11]. The radiolabelling method seems to offer an attractive alternative in some situations, although leaching of the isotope can produce misleading results [6].

Crystal violet (CV) staining, which is commonly used for the indirect quantification of adherent cells and amount of biofilm formed by *Candida* on abiotic surfaces, is a quick and cheap method. It involves a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix, [8, 12] and dissolves easily in acetic acid.

The aim of this study is to develop a quick and simple technique to assess the number of *Candida* adherent to epithelial cells, based on the quantification of crystal violet absorbance.

MATERIAL AND METHODS

Yeasts and growth conditions

Candida species used in this study were *Candida albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750), obtained from the American Type Culture Collection. Strains were kept frozen at – 80 °C in Sabouraud dextrose broth (SDB; Liofilchem, Italy) containing 5% (v/v) glycerol. *Candida* species were subcultured on Sabouraud dextrose agar (SDA; Liofilchem, Italy) for 24 h and then grown in SDB for 18 h at 37 °C at 120 rpm. After incubation, yeasts were harvested at 8000 rpm for 5 min. Cells resuspended in phosphate-buffered saline (PBS, 0.01 mol/l, pH 7.5) were enumerated using a haemocytometer (Boeco, Germany) and the final concentration (specific to each assay) was adjusted with PBS.

Epithelial cells

A human urinary bladder epithelial cell line (TCC-SUP; DSMZ – German Collection of Microorganisms and Cell Cultures) was used. Cells were cultured at 37 °C in 5% CO^2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 15% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (P/S; Gibco, USA) in cell culture flasks. After

achieving 80% confluence, cells were detached using a 25% trypsin-EDTA solution (Gibco, USA) and cell concentration was adjusted to 1 x 10^6 cells/ml with fresh DMEM without P/S and added to each well of a six well plate. Wells were washed (x 2) with PBS prior to assay.

Silicone

Coupons (2 x 2 cm) were cut from a 1 mm layer of silicone (Neves e Neves, Trofa, Portugal). All coupons were cleaned by immersion in ultrapure water for 2 h, followed by immersion in ethanol 50% (v/v) for 4 h. After rinsing with ultrapure water and air-drying, the coupons were autoclaved for 15 min at 121 °C.

Adhesion assay

Yeast cells were suspended in PBS to final concentrations of 10^4, 10^5, 10^6, 10^7 and 10^8 yeast/ml. Then, 3 ml each cell suspension was added to each well of the plate for tissue culture containing either a confluent layer of epithelial cells or the silicone coupon. After incubation (2 h, 120 rpm, 37 °C) the wells were washed (x 2) with PBS to remove unattached yeasts. Yeast cells were quantified using the CV staining method and also light microscope observation. All procedures were repeated in triplicate in three separate assays.

Crystal violet assay

Crystal violet (3 ml, 1% [w/v] in water) was added to each well containing the epithelial cells with adherent yeasts and allowed to stain for 5 min. The wells were then washed (x 3) with PBS. To remove CV from the epithelial cells, 3 ml ethanol: acetone (1: 1) was added to the wells and removed immediately. Acetic acid (33%, 3 ml) was added to each well and absorbance was read at 570 nm. Wells containing epithelial cells without yeasts were used as controls. Mean absorbance of yeasts was expressed as absorbance per area of each well.

Candida adherent to silicone were quantified according to the method of Henriques *et al*. [**13**]. Briefly, the coupons containing adherent yeasts were removed from each well and immersed for 5 min in a new well plate containing 3 ml methanol. After discarding the methanol, the coupons were allowed to dry at room temperature. Crystal violet (3 ml) was added to each well and allowed to stain for 5 min. Coupons were then removed to a new well, washed with ultrapure water and immersed in 3 ml acetic acid (33%) to dissolve the stain. Coupons without yeasts were used as controls. Absorbance of the resultant acetic acid solution was read at 570 nm. Mean absorbance of yeasts was expressed as absorbance per unit area of the coupon.

Microscope observation

Epithelial cells and coupons with adherent yeasts were treated as described above, but without acetic acid. A duplicate of each plate was performed. *Candida* attached to TCC-SUP were quantified using an inverted light microscope (Nikon Diaphot, x 400 magnification). Ten fields were randomly observed in each well. As the samples were set up in triplicate for each experiment, the mean number of yeasts per 30 fields was expressed as number of cells per unit area of the well. *Candida* cells with small daughter cells were regarded as one cell.

Statistical analysis

Results obtained were analysed using the SPSS (Statistical Package for the Social Sciences) program. One-way ANOVA with Bonferroni test was used to compare the number of adherent cells of the four strains. $P < 0.05$ was considered significant.

RESULTS

The method proposed in this study involves CV staining of *Candida* adherent to an epithelial cell monolayer. As cells, yeasts and epithelium are all stained with CV (Figure 3.1 a) it was necessary to develop a technique (using a mixture of ethanol/ acetone) that permitted the removal of CV from epithelial cells (Figure 3.1 b), allowing it to remain in the *Candida* cells (Figure 3.1 c). It was then possible, using acetic acid, to remove CV from the *Candida* and read the absorbance of the solution obtained.

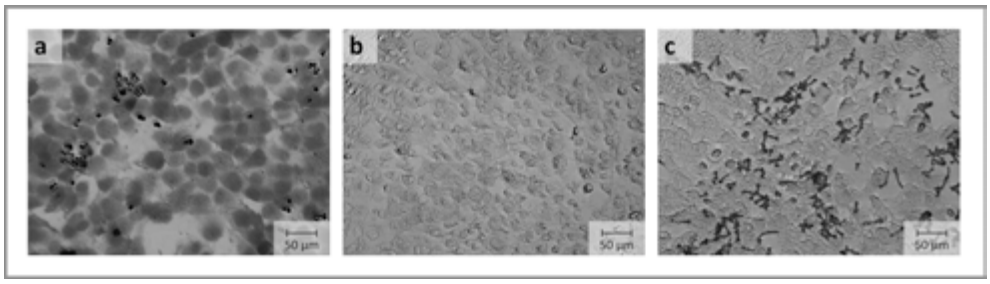


Figure 3.1: Phase contrast images of the steps of the proposed method: a) yeasts and TCC-SUP cells stained with CV only; b) TCC-SUP cells distained with ethanol and acetone; c) Yeast cells stained strongly with crystal violet and TCC-SUP cells distained with ethanol and acetone (original magnification x 200).

After the confirmation, by microscopy, of the applicability of this method, it was necessary to validate the technique by assessing and comparing adhesion using traditional enumeration of adherent yeast cell by microscope observation. The relationship between CV absorbance and the number of *Candida* attached to epithelial cells is presented in Figure 3.2. The values obtained were r^2 = 0.9995 for *C. albicans*, r^2 = 0.9997 for *C. tropicalis*, r^2 = 0.9724 for *C. glabrata* and r^2 = 0.9997 for *C. parapsilosis*.

The method proved adequate for the detection of *Candida* attachment at high yeast numbers, specifically above 1 x 10^5 cell/cm^2 for *C. albicans* (Figure 3.2 a), 2 x 10^5 cell/cm^2 for *C. tropicalis* (Figure 3.2 b), 3 x 10^4 cell/cm^2 for *C. glabrata* (Figure 3.2 c) and 1 x 10^4 cell/cm^2 for *C. parapsilosis* (Figure 3.2 d).

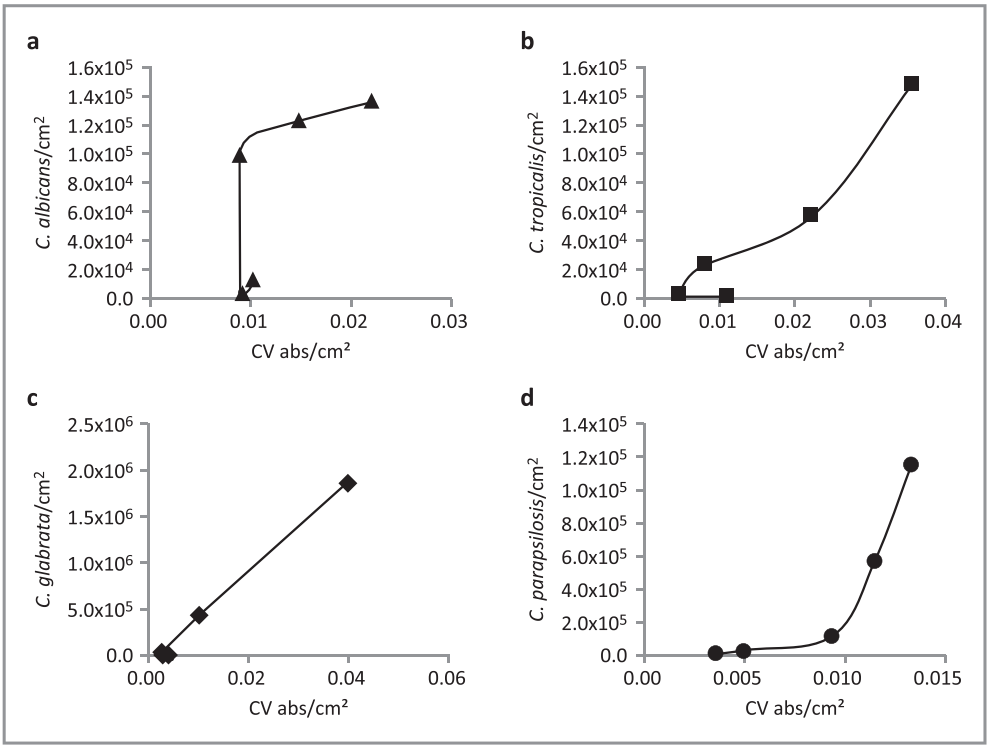


Figure 3.2: Relationship between the number of a) *Candida albicans*, b) *C. tropicalis*, c) *C. glabrata* and d) *C. parapsilosis* adherent to TCC-SUP epithelial cells, and the corresponding CV absorbance (CV abs) at 570 nm. The adherent *Candida* species were expressed as yeast number or CV absorbance per area of each well. All procedures were performed in triplicate in three separate assays.

Different *Candida* species have different sizes and absorb distinct amounts of dye, which does not allow comparison of the level of adhesion through direct CV absorbance readings. However, standardisation of the results is possible using respective equation curves for each species.

After the implementation of the methodology, the extent of adhesion of the different *Candida* species to TCC-SUP epithelial cells and also to silicone was determined (Figure 3.3). As shown in Figure 3.3 a, there were significant differences in the number of yeasts adherent to epithelial cells among the different concentrations tested for each *Candida* specie, except for *C. parapsilosis*. On silicone, the differences were significant for all *C. albicans* and *C. parapsilosis* inocula concentrations but only for the highest values in *C. glabrata* and *C. tropicalis* (Figure 3.3 b). All *Candida* species adhered to a greater extent to epithelial cells than to silicone.

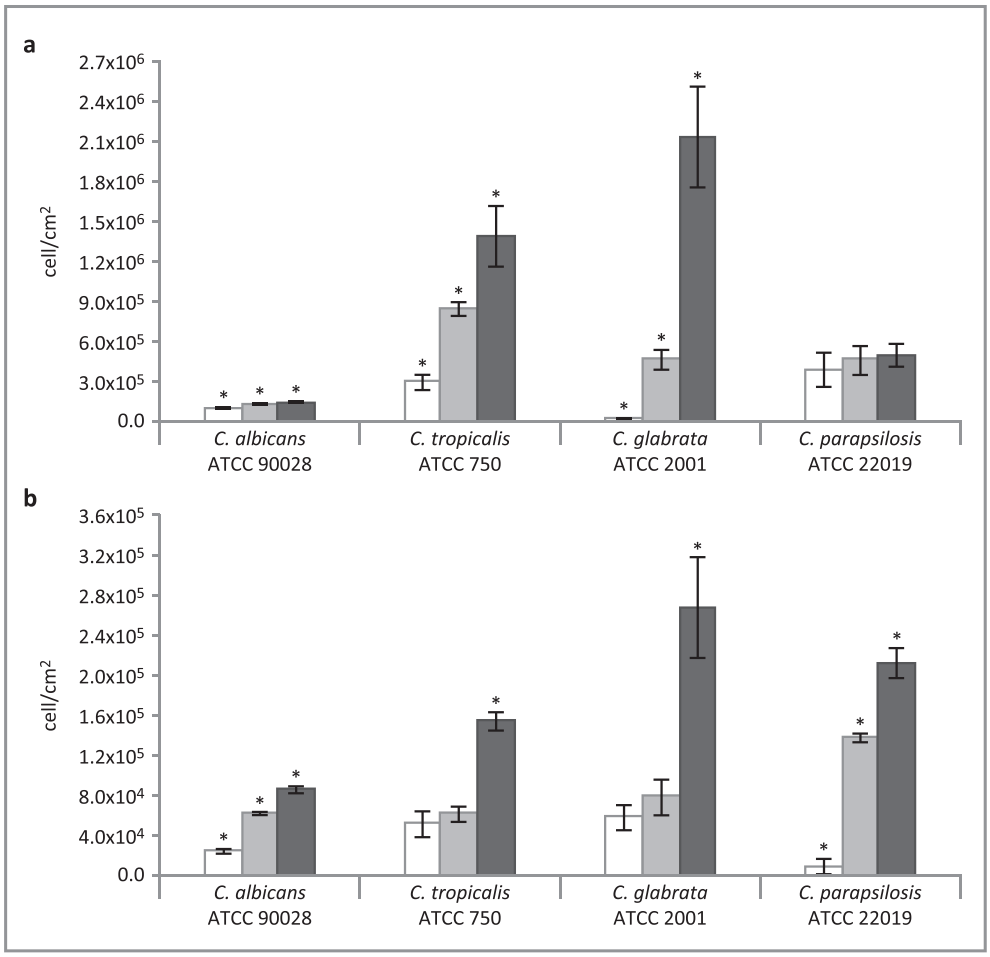


Figure 3.3: *Candida* species cells adherent to a) TCC-SUP epithelial cells and b) silicone measured by crystal violet absorbance reported as cell/cm^2. Data are the average of three measurements (+SD). The initial cell density: 10^6 (▒), 10^7 (▓) and 10^8 cell/ml (■). *P < 0.05 between the different inoculums for the same species.

Considering the difference between species, it is possible to observe (Figure 3.3) that *C. glabrata* followed by *C. tropicalis* adhered significantly (P < 0.05) more than other *Candida* species (initial cell density: 10^8 yeast/ml) to epithelial cells. *C. albicans* adhered less than other yeasts to both surfaces at 10^8 yeasts/ml (P < 0.05).

The methodology proposed proved efficient in demonstrating the *in vitro* adherence of *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750) to TCC-SUP.

DISCUSSION

Most *Candida* infections are associated with adhesion to implanted medical devices or to host epithelial cell surfaces [14]. *In vitro* adherence studies of *Candida* on different surfaces are well established [3, 4, 8, 10, 15]. One quantification method is direct enumeration by microscopy, which has been used widely to determine the extent of yeast adhesion to epithelial cells [16]. Although this technique permits visualisation of the yeast cells adherent to individual epithelial cells, it is very time-consuming [11]. Other techniques described to evaluate *Candida* adhesion to biological and inert surfaces include indirect immunofluorescence [17], fluorescence-labelled cytometry [18], radioisotope analysis [19] and photometric quantification [20]. However, most of these do not balance accuracy, speed, reproducibility and cost effectiveness [6]. The method described here is based on CV staining to quantify microbial adhesion and biofilm formation on inert surfaces [13, 21]. However, the application of this method to assess adhesion to epithelial cells is not straightforward as both epithelial and yeast cells absorb CV dye (Figure 3.1 a). It is possible to circumvent this problem by using a mixture of ethanol/ acetone to remove the stain from the epithelial cells (Figure 3.1 b) but not the *Candida* cells (Figure 3.1 c).

Figure 3.2 shows that it is possible to establish a direct relationship between the number of *Candida* adherent to TCC-SUP cells and CV absorbance, and were obtained for initial inocula concentrations of 10^6, 10^7 and 10^8 yeast/ml. According to Henriques *et al*. [11] there is no detectable yeast attachment at concentrations below 10^4 yeast/ml, and 10^7 yeast/ml is the most frequently used *Candida* inoculum concentration. The high correlation between CV absorbance values and the number of *Candida* cells obtained by microscopy confirms the utility of determining attached cell numbers through the corresponding absorbance reading [16, 22].

The extent of adhesion of *C. albicans* 90028, *C. glabrata* 2001, *C. parapsilosis* 22019 and *C. tropicalis* 750 to TCC-SUP cells and to silicone

was also determined using the proposed method (Figure 3.3). As *Candida* species differ on size, their absorbance value was standardised (according to each species curve) in order to permit comparison. It was noticed that different inoculum concentrations gave significantly different ($P < 0.05$) numbers of *Candida* adherent to the epithelial cells; an observation made by others [23-25] who report that the attachment of *Candida* species to epithelial cells gradually increases as the ratio of yeasts to epithelial cells, in incubation mixtures, is raised from 10: 1 to 10000: 1.

Differences were detected in the adherence of *Candida* species to TCC-SUP cells and silicone. All *Candida* were more prone to adhere to TCC-SUP than to silicone (Figure 3.3). Sohn *et al*. [3] described the comparison of adhesion of *C. albicans* to the human colorectal carcinoma cell line Caco-2 and to epidermoid vulvovaginal A431 cells and to polystyrene, reporting that *C. albicans* adherence is high to polystyrene and both epithelia.

C. albicans and *C. parapsilosis* adhered to a similar extent on both surfaces at an initial concentration of 10^8 cells/ml, but less than the other yeasts studied. Tamura *et al*. [26] investigated the adherence of *C. albicans* and *C. parapsilosis* to urinary catheters made of latex or silicone and found that adhesion of *C. albicans* was significantly greater than *C. parapsilosis* on latex, but similar on silicone. Furthermore, De Bernardis *et al*. [1] observed that from all the non-*C. albicans* species (NCAC) studied, *C. parapsilosis* seemed to be the least virulent, which might be consistent with lower adherence to epithelial cells.

Owing to the increasing prevalence of NCAC species, especially in immunosuppressed patients, more insight about virulence factors associated with these species is required. However, relatively little is known about the mechanisms of NCAC adhesion to epithelium or about factors affecting the adhesion process [11]. *Candida tropicalis* is a common species related to nosocomial candidemia and candiduria, and *C. glabrata* is now emerging as an important agent in both mucosal and bloodstream infections [14, 27]. Nevertheless, the adhesion mechanism of these species to different surfaces remains unclear [11, 14].

From Figure 3.3 a, it is possible to see that *C. glabrata* and *C. tropicalis* ($P < 0.05$) adhered in greater numbers than did other yeasts (initial cell density: 10^8 cell/ml) to epithelial cells. Strain differences were noted in the ability of *C. glabrata* to adhere to oral epithelial cells, synthesise phospholipases and trigger cytokine responses [28-30]. According to Shin *et al*. [31] biofilm occurred most frequently in isolates of *C. tropicalis*, followed by *C. parapsilosis*, *C. glabrata* and *C. albicans*. Virulence of *C. tropicalis* may be due to its greater adhesion to different surfaces and its ability to secrete moderate amounts of proteinase and filamentous forms compared to other NCAC species [14, 19, 32].

In conclusion, the proposed methodology is both easy to execute and cheap, and is reproducible in assessing *Candida* adhesion to TCC-SUP cells. It is a valuable methodology to discriminate the adhesive capacity of different *Candida* species isolates to different epithelial cells, and may contribute to research on the virulence of *C. albicans* and NCAC species.

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CHAPTER 4

Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients

ABSTRACT

Candida tropicalis has been reported to be one of the *Candida* species, which is most likely to cause bloodstream and urinary tract infections in hospitalized patients. Accordingly, the aim of this study was to characterize the virulence of *C. tropicalis* by assessing antifungal susceptibility and comparing the expression of several virulence factors. This study was conducted with seven isolates of *C. tropicalis* from urine and blood cultures and from central venous catheter. *Candida tropicalis* ATCC750 was used as reference strain. Yeasts adhered (2 h) to epithelial cells and silicone and 24 h biofilm biomass were determined by crystal violet staining. Pseudohyphae formation ability was determined after growth in fetal bovine serum. Enzymes production (haemolysins, proteinases, phospholipases) was assessed by halo formation on agar plates. Susceptibility to antifungal agents was determined by E-test. Regarding adhesion, it can be highlighted that *C. tropicalis* strains adhered significantly more to epithelium than to silicone. Furthermore, all *C. tropicalis* strains were able to form biofilms and to express total haemolytic activity. However, proteinase was only produced by two isolates from urine and by the isolates from catheter and blood. Moreover, only one *C. tropicalis* (from catheter) was phospholipase positive. All isolates were susceptible to voriconazole, fluconazole and amphotericin B. Four strains were susceptible-dose dependent to itraconazole and one clinical isolate was found to be resistant.

Keywords: *Candida tropicalis*; virulence factors; secretion of enzymes; biofilm; adhesion.

Negri M, Martins M, Henriques M*,* Svidzinski T, Azeredo J, and Oliveira R*.* Examination of potential virulence factors of *Candida tropicalis* clinical isolates from hospitalized patients. Mycopathologia 2010; 169 (3): 175-182.

INTRODUCTION

Fungal hospital infections (FHI) incidence has increased significantly over the last decades. *Candida* species are the most frequently isolated fungi, corresponding to approximately 80% of FHI, being the fourth responsible for blood stream infection and the overwhelming majority responsible for urinary tract infections [1-3].

Usually, *Candida tropicalis* is considered the third *Candida* species most frequently isolated from urine cultures [4, 5]. Moreover, in a recent epidemiological study conducted in 12 Brazilian medical centres, *C. tropicalis* was the second most frequent *Candida* species, accounting for 20–24% of all candidemia [2, 6]. Additionally, *C. tropicalis* is often found in patients admitted in intensive care units (ICUs), especially in patients with cancer or/and requiring prolonged catheterization, or receiving broad-spectrum antibiotics [2, 4, 6-8].

Several virulence factors seem to be responsible for *C. tropicalis* infections, which present high potential for dissemination and mortality [8, 9]. Adhesion to host surfaces (epithelial cells and medical devices), as well as biofilm formation, are considered the first step to initiate *Candida* infection [10, 11]. Furthermore, secretion of enzymes (proteinases and phospholipases), as well as haemolytic activity, are recognized as important factors in tissue invasion [10-14].

Hence, the aim of this study was to assess and compare the expression of different virulence factors (enzymes secretion, adhesion and biofilm formation and pseudohyphae production) by several *C. tropicalis* clinical isolates. Moreover, antifungal susceptibility was also determined in order to deeply characterize the virulence of *C. tropicalis*.

MATERIALS AND METHODS

Isolates

This study was conducted with seven isolates of *C. tropicalis*: five obtained from candiduria, one from candidemia and one from a central venous catheter (CVC) and all were from patients admitted to ICUs at the University Hospital (UH) in Maringá, Paraná, Brazil. *Candida tropicalis* ATCC 750 was used as reference strain.

Isolation and identification

Yeasts were isolated according to hospital routine methods. To perform hemoculture, one volume of blood was inoculated in 10 volumes of Trypticase Soy Broth (Difco, Detroit, Michigan, EUA) and incubated in the automatic BACTEC (Becton–Dickinson Microbiology Systems, Sparks, MD) system. Urine was spread using a calibrated loop (10 μl) on CLED medium agar plates (Difco) and incubated at 37 °C for 48 h. CVC isolated yeasts underwent a well established technique [15]. Briefly, CVC was rolled on blood agar plates (Difco) and incubated at 35 °C for 72 h. After yeast growth, they were subcultured in CHROMagar *Candida*® (CHROMagar, BioMerieux, Paris, France) to assess the purity of the culture and the colour of the colonies. From this selective and differential medium, yeasts were identified by three methods: the MicroScan rapid yeast identification panel (Dade Behring Inc, CA, USA), the classical biochemical method [16] and molecular identification.

Candida DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, IZASA, Lisbon, Portugal) according to the manufacturer's instructions. DNA content was determined by spectrophotometry readings at 260 nm. Aliquots of 10 μl were analysed by electrophoresis in a 0.8% agarose (Bio-Rad, Lisbon, Portugal) gel in 1 x TBE buffer (Bio-Rad) and visualized with a UV transilluminator after ethidium bromide (Bio-Rad) staining (0.5 mg/ml). To assess *Candida* speciation, a polymerase chain reaction (PCR) method previously described [17] was used. Specific primers for the genomic

sequences of DNA topoisomerase II of *C. albicans, C. dubliniensis, C. tropicalis* (genotypes I and II), *C. parapsilosis* (genotypes I and II), *C. krusei, C. kefyr*, *C. guilliermondii* and *C. glabrata* were used.

Adhesion and biofilm Formation

Yeast cells were grown at 37 °C, 120 rpm for 18 h on Sabouraud Dextrose Broth (SDB; Difco) and Phosphate saline buffer (PBS)—washed suspensions of each yeast culture were resuspended in RPMI 1640 (Sigma, Saint Louis, Missouri, USA) to a final concentration of 1.0 x 10^7 cells/ml. Then, 3 ml of the suspension was added to each well of a 6-well plate containing either a confluent layer of TCC-SUP human urinary bladder epithelial cell line (DSMZ—German Collection of Microorganisms and Cell Cultures) or a silicone coupon (2 x 2 cm) (Neves e Neves, Trofa, Portugal). All procedures were performed in triplicate and repeated in three separate assays.

Quantification of adhered yeast cells

After 2 h of incubation (120 rpm, at 37 °C), the wells were washed twice with PBS to remove unattached yeasts. Yeast cells were quantified using the crystal violet (CV) staining method.

Epithelial cells

Three millilitres of CV stain (1%) was added to each well containing the epithelial cells with adherent yeasts and allowed to stain for 5 min. Then, the wells were washed three times with PBS. In order to remove the CV stain from the epithelial cells, 3 ml of ethanol: acetone (1: 1) was added to each well and removed immediately. Three millilitre of acetic acid (33%) was added to each well and the absorbance of the final solution was read at 570 nm in a microtiter plate reader (Bio-Tek® Synergy HT, IZASA). Wells containing epithelial cells without yeasts were used as controls. The mean absorbance of CV retained by yeasts was expressed as absorbance per unit of well area.

Silicone

Candida cells adhered to silicone were quantified according to Henriques *et al.* [18]. Briefly, the coupons containing adherent yeasts were removed from each well and immersed for 5 min in new well plates containing 3 ml of methanol. After withdrawing the methanol, the coupons were allowed to dry at room temperature. Then, 3 ml of CV (1%) was added to each well and allowed to stain for 5 min. Coupons were transferred to a new well, washed with ultrapure water and immersed in 3 ml acetic acid (33%) to dissolve the stain. Coupons without yeasts were used as controls. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader at 570 nm. The final number of cells attached, per coupon unit area, was determined using the mean absorbance and the respective calibration curves, previously established.

Biofilm biomass quantification

After 24 h of incubation (120 rpm, at 37 °C) *C. tropicalis* biofilm forming ability was assessed on inert materials through quantification of total biomass by CV staining as described above. Experiments were repeated in 3–5 independent assays.

Pseudohyphae formation

Pseudohyphae formation was defined as a cell bearing a rounded outgrowth with a length greater than or equal to the diameter of the parent cell, with a constriction at the base. The percentage of cells in pseudohyphae form, against blastopores, was determined by microscopy counting after 2 h of cell growth in a liquid medium containing equal volumes of RPMI 1640 (Sigma) and fetal bovine serum (GIBCO, New York, USA). In these experiments, 100 cells per field were examined. Each experiment was conducted in triplicate.

Proteinase and phospholipase secretion

Secretion of proteinases and phospholipases was detected by the formation of an opaque halo of degradation around the colonies grown in a specific agar plate, according to Ruchel [19] and Price *et al.* [20], respectively. An aliquot (5 μl) of a 1 x 10^8 cells/ml suspension prepared in distilled water was inoculated on proteinase agar medium (2% agar, 1.17% yeast carbon base, 0.01% yeast extract and 0.2% bovine serum albumin) pH 5.0 and on phospholipase agar (2% agar, 1% peptone, 2% glucose, 1 M NaCl, 5 mM $CaCl_2.2H_2O$ and 8% egg yolk) and the Petri dishes were incubated for 7 days at 37 °C. The enzymatic activity (Pz) was determined by the ratio between the colony diameter and the colony diameter plus the halo zone as described by Price *et al.* [20]. Each experiment was conducted in triplicate.

Haemolytic activity

Haemolysin production was evaluated using a modification of the plate assay described by Luo *et al.* [13]. Fresh cultured colonies of *C. tropicalis* were obtained after being spread on Sabouraud Dextrose Agar (SDA; Difco) (18–24 h). Then, a suspension was prepared in saline solution (0.9% NaCl) to reach 10^8 cells/ml, enumerated using a Neubauer chamber. Ten microliters of this suspension was spot-inoculated on sugar-enriched sheep blood (2% peptone, 1% agar, 7% fresh sheep blood, 3% glucose) and the plates were incubated at 37 °C. After 48 h they were classified as absent (no halo), partial, or total (completely transparent halo) haemolytic activity. The assay was conducted in quadruplicate on two separate occasions for each yeast isolate tested. A standard strain, *C. albicans* ATCC 90028, was used as control in each experiment.

Antifungal susceptibility test methods

The E-test method was used as recommended by the manufacturer with strips, provided by AB BIODISK (Solna, Sweden), having the following concentrations: from 0.002 to 256 μg/ml; for fluconazole (FLU) and from

0.002 to 32 μg/ml for itraconazole (ITR), voriconazole (VO) and amphotericin B (AMB). The minimum inhibitory concentrations (MIC) of drugs were determined on RPMI 1640 (Sigma) agar with 2% glucose. An inoculum suspension was adjusted to a turbidity of 0.5 McFarland standard (1×10^6 to 5×10^6 cells/ml) and was incubated at 37 °C for 48 h. MICs were read as the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip. Quality control was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) document M27-A3 [21], using *Candida krusei* ATTC 6258 and *C. parapsilosis* ATCC 22019. MIC interpretative criteria was performed according to the CLSI M27-A3 [21]: (a) FLU: MIC ≤ 8 μg/ml—susceptible (S); 16 < MIC < 32 μg/ml—susceptible-dose dependent (S-DD) and MIC > 64 μg/ml—resistant (R); (b) ITR: MIC ≤ 0.125 μg/ml—S; 0.25 < MIC < 0.5 μg/ml—S-DD and MIC ≥ 1 μg/ml—R; (c) AMB MIC > 1 μg/ml—R; (d) VO MIC ≥ 1 μg/ml—S; ≤ 2 μg/ml—R.

Statistical analysis

Results obtained were analysed using the SPSS (Statistical Package for the Social Sciences) program. One-way ANOVA with the Bonferroni test was used to compare the number of adherent cells of the several strains assayed. All tests were performed with a confidence level of 95%.

RESULTS

A total of seven *C. tropicalis* isolates was used in this study: five obtained from urine samples, one from blood samples and one from CVC, all from patients admitted to ICUs at the UH of Maringá, Paraná, Brazil. Additionally, all the methods (MicroScan rapid yeast identification panel, classical biochemical and molecular identification) used had identified *C. tropicalis* with 100% concordance (data not shown).

Regarding adhesion ability (Figure 4.1), it can be highlighted that all *C. tropicalis* adhered in a significantly greater extent ($P < 0.05$) to epithelial

cells than to silicone. Considering the differences among the isolates, it is possible to observe from Figure 4.1 that *C. tropicalis* L012, from CVC, adhered in a highest extent to epithelial cells (4.05 x 10^6 cells/cm^2) with $P < 0.001$ vs. strains 1, 16, 29, 69 and to silicone (9.37 x 10^5 cells/cm^2) with $P < 0.001$ vs. strains 1, 12, 69. Clinical isolates 29 and 69, from urine cultures, adhered to epithelial cells in lower number than other yeasts ($P < 0.05$ vs. L012) and the latter, as well as strain 12, adhered in lower extent than other yeasts to silicone (Figure 4.1).

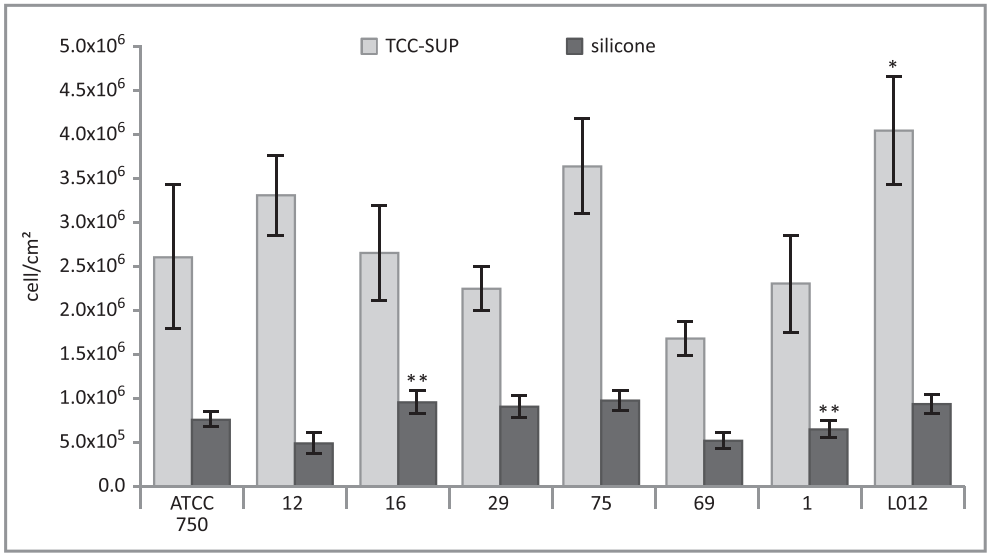

Figure 4.1: Number of *C. tropicalis* cells per cm^2 (cell/cm^2) (mean ± standard deviation) adhered to TCC-SUP epithelial cells and to silicone measured by crystal violet staining. * represents the statistical differences ($P < 0.05$) of adhesion extension to TCC-SUP between the strain L012 and strains 1, 16, 29, 69, ATCC 750; ** represents the statistical differences ($P < 0.05$) of adhesion extension to silicone of the strains 16 and 1 compared to the strains 29, 75, L012, ATCC 750.

As it can be observed in Figure 4.2, all *C. tropicalis* were able to form biofilms and strains did not present significant statistical differences.

Concerning *C. tropicalis* pseudohyphae formation and enzymatic activity (Table 4.1), it was possible to verify that all isolates produced a low percentage of pseudohyphae formation. Among all, isolates 12 and L012 presented the highest production of pseudohyphae formation (10 and 7%, respectively). Nevertheless, all isolates were able to express total

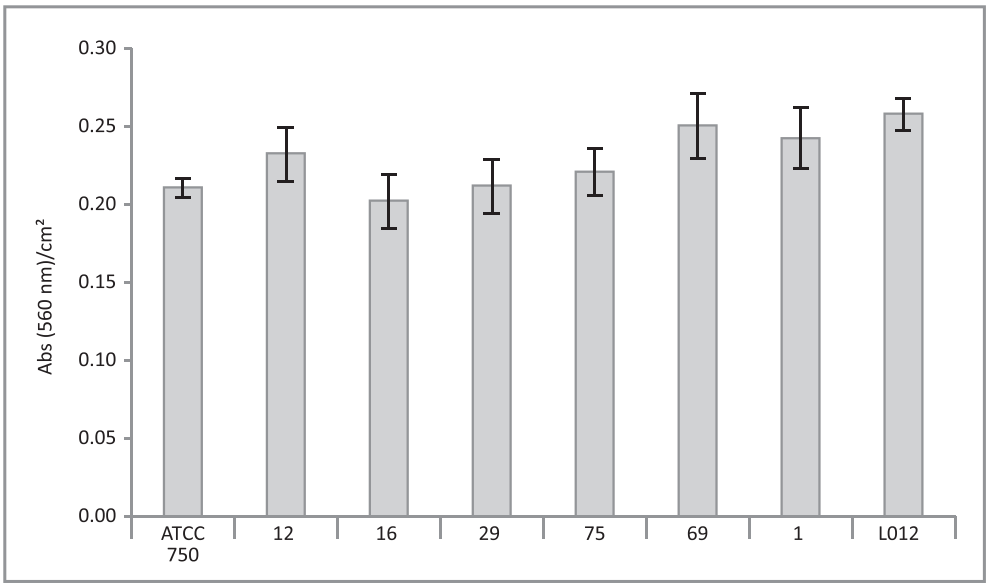

Figure 4.2: Biofilm biomass on silicone measured by crystal violet staining, expressed as absorbance/cm^2 [Abs (570 nm)/cm^2]. Mean ± standard deviation.

haemolytic activity on sheep-blood agar medium supplemented with glucose. However, proteinase was only produced by two isolates from urine and by the isolates from catheter and blood and only one *C. tropicalis* (from CVC) was phospholipase positive.

Table 4.1: Comparison of putative virulence factors of *C. tropicalis* from clinical isolate, including proteolytic (Pro), phospholytic (Phos) and haemolysis (Hemo) activity and ability for pseudohyphae formation

Isolates		Activity			Pseudohyphae formation
Sites	Strain	Pro[a]	Phos[a]	Hemo	%
Urine	12	1.00	1.00	Total	10
Urine	16	0.81	1.00	Total	4
Urine	29	0.85	1.00	Total	3
Urine	75	1.00	1.00	Total	4
Urine	69	1.00	1.00	Total	1
CVC	L012	0.89	0.66	Total	7
Blood	1	0.69	1.00	Total	2

[a] Values of enzyme activity, according to Price et al. [20]:
Pz = 1.00 means that the test strain is negative for enzymatic activity, while a value of Pz < 1.00 means that the test strain is positive for enzyme activity

The levels of *C. tropicalis* isolates antifungal susceptibility are shown in Table 4.2. It is possible to observe that all isolates showed susceptibility (S) to VO, FLU and AMB. The largest percept of S-DD was observed for ITR in four strains and one clinical isolate from urine was found to be resistant (MIC = 1 µg/ml).

Table 4.2: *In vitro* susceptibility of *C. tropicalis* from clinical isolates for amphotericin B (AMB), voriconazole (VO), itraconazole (ITR) and fluconazole (FLU)

Isolates		Susceptibility category (MIC µg/ml) [a]			
Sites	Strain	AMB	VO	ITR	FLU
Urine	12	S (0.5)	S (0.032)	S (0.064)	S (0.5)
Urine	16	S (0.25)	S (0.032)	SDD (0.38)	S (0.5)
Urine	29	S (0.38)	S (0.023)	SDD (0.38)	S (0.5)
Urine	75	S (1)	S (0.064)	SDD (0.5)	S (0.125)
Urine	69	S (0.125)	S (0.125)	R (1)	S (2)
CVC	L012	S (0.003)	S (0.023)	SDD (0.25)	S (1)
Blood	1	S (0.047)	S (0.012)	S (0.032)	S (0.25)

[a] MIC interpretative criteria was performed according to the CLSI M27-A3 [21]

DISCUSSION

Nosocomial infections by Non-*Candida albicans Candida* (NCAC) species such as candidemia and candiduria have emerged as an increasing problem during the last two decades [22, 23]. Moreover, *C. tropicalis* appears to display higher potential for dissemination and mortality and possesses several virulence factors that can enhance the progression of infections than *C. albicans* and other NCAC species [6, 8, 24].

In the present study, the results of morphological and biochemical tests were in agreement with molecular identification. Morphological, serological, or biochemical tests have been used, along the years, for the identification of *Candida* species. However, misidentification has been reported specially in relation to *C. tropicalis* [3, 10]. Recently, molecular-based techniques, which are simpler and more efficient than the conventional tests, have been adapted to the identification of pathogenic microorganisms. In particular, due to its speed, reproducibility, high sensitivity and specificity, PCR tests have been increasingly used in laboratories for identification of several fungal species [3, 10, 17].

Concerning adhesion ability, it was possible to verify that all *C. tropicalis* adhered in a significantly higher extent ($P < 0.05$) to epithelial cells than to silicone. Sohn *et al.* [25] compared the ability of *C. albicans* to adhere to the human colorectal carcinoma cell line Caco-2 and epidermoid vulvovaginal A431 cells and to polystyrene and reported that *C. albicans* adheres in high extent to polystyrene and both epithelia. According to Bendel and Hostetter [26], the extent of adhesion to the human epithelia cell line HeLa S3 did not differ between *C. albicans* and *C. tropicalis* despite both species having distinct mechanisms for this process. Nevertheless, the present results clearly show differences in the adhesion ability among the different isolates and hence adhesion of *C. tropicalis* to biotic and abiotic surfaces is strain dependant. Different intra-species adherence ability was also reported by other authors for other *Candida* species [9, 27-29].

We did not find a clear relation between pseudohyphae formation and adhesion capability. Although *C. tropicalis* L012, from CVC, and strain 12, from urine, adhered in higher extent to epithelial cells presenting also more pseudohyphae formation, the strain 75 exhibited an intermediate pseudohyphae formation and high adhesion ability. On the other hand, clinical isolates 29 and 69, from urine cultures, adhered to epithelial cells and displayed pseudohyphae formation in a lower extent than the other strains. This evidences the current lack of knowledge concerning the adhesion mechanisms of NCAC species to epithelium, as well as about the factors affecting the adhesion process [18].

Adhesion of *Candida* spp. to hard materials or host cells has been considered as an early step in biofilm formation [30, 31]. Nevertheless, it is important to highlight that, although strains 69 and 12 adhered in small number than other yeasts to silicone (Figure 4.1) they showed higher ability to form biofilms on this material (Figure 4.2). This result is consistent with other studies [14, 32], reporting differences between adhesion and biofilm formation abilities on polystyrene and poly (vinyl chloride) (PVC) surfaces under static conditions, which also suggest that adhesion and biofilm formation of *C. tropicalis* are two distinct phenomena.

All *C. tropicalis* strains tested were able to form biofilms on silicone, which has important clinical impact once biofilm-associated infections are difficult to treat, representing a source of reinfections [23, 33]. Previous works also reported that *C. tropicalis* can form extensive *in vitro* biofilms in PVC catheters [34] and polystyrene surfaces [33].

The infective ability of yeasts depends on specific virulence mechanisms that confer the ability to colonize host surfaces, to invade deeper host tissue or to evade host defences [9, 35, 36]. During the pathogenic process many virulence attributes may be involved including production of extracellular proteinases and phospholipases, as well as haemolytic activity [10-14, 37, 38]. In this study, all isolates were able to express total haemolytic activity. Manns *et al.* [12] demonstrated that *C. albicans* produced haemolytic activity and Luo *et al.* [13] observed that NCAC species are capable of producing one or more types of haemolysins *in vitro* with differences among species. Moreover, they observed that *C. tropicalis* was able to produce complete haemolysis after 48 h, corroborating the results obtained herein.

In the present case, only few isolates were proteinase and phospholipase positive, corroborating the results of other authors [10, 39]. In opposition, Kumar *et al.* [38] detected 100 and 72.9% of proteinases and phospholipase producers, respectively, among *Candida* species isolated from pulmonary tuberculosis patients. According to these results, proteinase and phospholipase expression can vary according to *Candida* species, strain and the site of isolation. Furthermore, although the methods used to test the presence of these enzymes are simple and fast they are not excessively accurate, specially compared with molecular methods that can detect gene expression [24, 36].

Concerning antifungal susceptibility results, all isolates were susceptible to VO, FLU and AMB and for four strains were S-DD for ITR. However, one clinical isolate showed to be resistant to ITR. These results are comparable to those reported in the literature, with slight differences that were dependent on the underlying disease and the *Candida* species involved in the infection [10, 14, 40, 41].

Though some authors have already assessed some *C. tropicalis* virulence factors, this work gathers, for the first time, the most important ones: secretion of enzymes, pseudohyphae formation, adhesion (to epithelial cells and silicone), biofilm formation and antifungal susceptibility. Despite it was not possible to establish a relation among the virulence factors assayed, it is interesting to notice that the strain isolated from CVC (L012) presented higher levels of all these factors. Furthermore, all clinical isolates presented one or more virulence factors.

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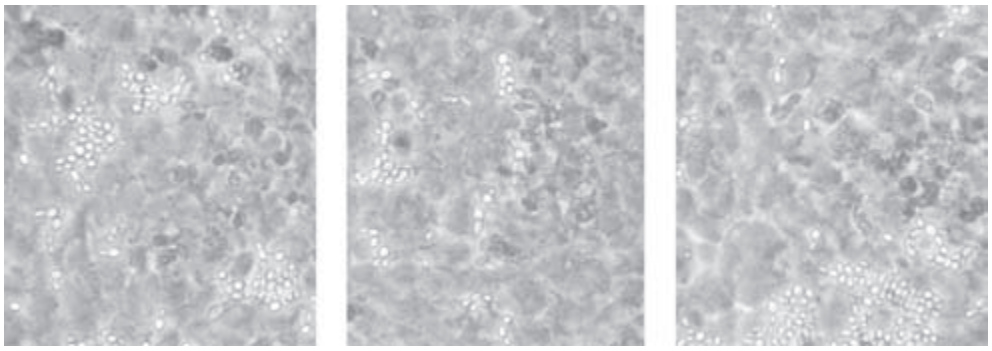
CHAPTER 5

An in vitro evaluation of *Candida tropicalis* infectivity using human cell monolayers



ABSTRACT

The aim of the present study was to investigate the interaction of *Candida tropicalis* with three different human cell lines: TCC-SUP (epithelial cells from urinary bladder); HeLa (epithelial cells from cervical carcinoma); Caco-2 (epithelial cells from colorectal adenocarcinoma). In particular to assess the degree of cell damage and activity reduction induced by *C. tropicalis* adhesion and the role of *SAPT* gene expression in this process. Two *C. tropicalis* strains were used in this study, the reference strain ATCC 750 and a clinical isolate from urine (U69). The ability of *C. tropicalis* to adhere to a confluent layer of human cells was determined using an adaptation of the crystal violet staining method; cell damage and cell activity inhibition induced by the adhesion of *C. tropicalis* were assessed by LDH and MTS reduction, respectively. *Candida tropicalis* aspartyl proteinase (*SAPT*) gene expression was determined by real-time PCR. *Candida tropicalis* strains were able to adhere to the different human cells, although, in a strain and cell dependent manner. Concerning cellular response to *C. tropicalis,* the highest cell activity inhibition was obtained for Caco-2, followed by TCC-SUP and HeLa cells. The highest percentage of cell damage (around 14%) was observed for TCC-SUP in contact with the U69 isolate and for Caco-2 in contact with the reference strain. Real time PCR analysis revealed a wide range of expression profiles of *SAP* genes for both *C. tropicalis* strains in contact with the different types of epithelial cells. *SAPT3* was the gene expressed at the highest level for both *C. tropicalis* strains in contact with the three human epithelial cell lines. It is important to highlight that human cells response to *C. tropicalis* adhesion, as well as *SAP*s production, is strain and epithelial cell line dependent.

Keywords: Adherence, *Candida tropicalis*; fungal infection, human pathogenic fungi, aspartyl proteinase

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INTRODUCTION

Adhesion to host surfaces, like human epithelial cells, and secretion of hydrolytic enzymes are considered important factors for *Candida tropicalis* virulence [1-4]. The capacity of *C. tropicalis* to adhere and to infect host cells becomes a serious problem when patients are in intensive care units (ICU), mainly because *C. tropicalis* is associated with higher dissemination potential and mortality, particularly in oncological patients [5-7]. In order to clarify the infectivity of *Candida* species, several *in vitro* studies have been performed using human cell monolayers from urinary, vaginal and intestinal epithelia [3, 8, 9].

In addition, hydrolytic enzymes such as aspartyl proteinases (Saps) are known to facilitate invasion and colonization of tissues by disrupting the host mucosal membranes and by degrading important immunological and structural defence proteins [1, 10]. It is also recognized that *C. tropicalis* possesses at least four genes encoding Saps, and these are designated *SAPT1, SAPT2, SAPT3* and *SAPT4* [1]

Since, *C. tropicalis* colonizes specific body sites such as the gastrointestinal and urinary tract and is associated with severe and invasive candidosis [11], it is important to understand the mechanisms of interaction between these epithelia and *C. tropicalis*. So, the aim of this work was to study the effect of *C. tropicalis* colonization of different human epithelial cells and the levels of *SAP*s genes expression by *C. tropicalis* when interacting with different human cells.

MATERIALS AND METHODS

Yeasts and growth conditions

Two strains of *C. tropicalis* were used in this study, one reference strain from the American Type Culture Collection (ATCC 750) and one isolate

(U69) obtained from a urine sample of a 84 years old female patient with candiduria admitted to the intensive care of an oncologic unit and belonging to the archive collection of the University Hospital in Maringá, Paraná, Brazil. For each experiment, strains were inoculated in Sabouraud dextrose broth (SDB; Merck, Germany) and incubated for 18 h at 37 °C under agitation at 120 rpm. After incubation, cells were harvested by centrifugation at 8000 × g for 5 min at 4 °C and washed twice with Phosphate Buffer Solution (PBS; pH 7.5; 0.01 mol/l).

Human epithelial cells line

To assess the adhesion ability of *C. tropicalis* to human epithelial cells, the following cell lines were used as models: (i) TCC-SUP cells, derived from human urinary bladder epithelial cells (DSMZ - German Collection of Microorganisms and Cell Cultures); (ii) HeLa cells, derived from a human cervical carcinoma, donated by "Instituto Gulbenkian de Ciência", Lisbon, Portugal; (iii) Caco-2 cells derived from a human colorectal adenocarcinoma cell line (ATCC HTB-37), kindly donated by Carla Nunes, Department of Biochemistry, Faculdade de Farmácia da Universidade de Coimbra, Coimbra, Portugal. Cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (D-MEM; Gibco, USA) containing 10% of fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (P/S; Gibco, USA). After achieving 80% of confluence, cells were detached using a 25% trypsin-EDTA (Gibco, USA) solution and cell concentration was adjusted to $1x10^6$ cells/ml with fresh D-MEM without P/S and added to a 24-well plate. Prior to the adhesion assays, the wells were washed two times with PBS.

Adhesion assay

The yeast cells were suspended in D-MEM, without phenol, to a final concentration of 1×10^7 yeast/ml using a Neubauer chamber (Boeco, Germany). Then, 1 ml of this suspension was added to each well of the 24-well plate covered with a confluent layer of a human epithelial cell line. After 2 h of incubation at 37 °C under 5% CO_2, each well was washed once with PBS to remove unattached yeasts.

Candida tropicalis quantification

The adhered yeasts were quantified using the crystal violet (CV) staining method, according to Negri *et al.* [12]. The mean absorbance of yeasts was expressed as the absorbance per area of each well and standardized by number of adhered yeasts per area of each well using *C. tropicalis* standard curve [3, 12]. All the procedures were repeated in triplicate in at least three separate assays.

Determination of percentage of inhibition of cell activity

After the washing step with PBS, the remaining adhered yeasts were killed by incubating the well plates for 2 h and by adding a 1% amphotericin B (AB) solution (Sigma, USA, 250 µg/ml) in D-MEM without phenol at 37 °C and 5% CO_2. Then, the AB solution was discarded and the epithelial cells activity was determined using the CellTiter 96® (MTS; Promega, USA) assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H–tetrazolium] with 1% AB in D-MEM without phenol. MTS is bioreduced by human epithelial cells into a formazan product that is soluble in tissue culture medium. So, after 2 h of incubation at 37 °C in the dark, the absorbance of the formazan was measured at 490 nm. A control was performed by measuring the cellular activity of human cells grown in the same conditions but in the absence of yeast cells. The effect of *C. tropicalis* in human epithelial cells was expressed as the percentage of inhibition of cell activity standardized by the number of adhered yeast cells, with MTS control corresponding to 100% of human cells activity, according to the following equation:

$$\%\,[\text{inhibition of cell activity}] = 100\ X \left\{ \frac{\left[\frac{[\text{MTS}]_{\text{control}} - [\text{MTS}]_{\text{cell activity with } \textit{C. tropicalis}}}{[\text{MTS}]_{\text{control}}} \right]}{\text{number of adhered yeasts}} \right\}$$

All the procedures were repeated in triplicate in at least three separate assays.

Epithelial cells damage assay

The release of lactate dehydrogenase (LDH) by epithelial cells into the culture medium was used as a measure of cell damage. The LDH concentration in the medium was measured after 2 h of adhesion using the CytoTox-ONE™ kit (Promega, Madison, USA) following the manufacturer's instructions. Two controls for LDH activity were prepared namely, (i) epithelial cells grown in the absence of *Candida* and (ii) yeast cells as sole culture. The LDH concentrations of both controls were subtracted from the LDH released by epithelial cells infected with yeasts. The effect of *C. tropicalis* on epithelial cells was expressed as the percentage of LDH released per number of adhered yeast cells, considering 100% the concentration of LDH released by a completely killed epithelial cell monolayer (using the killing buffer provided with the kit), according to the following equation:

$$\%[\mathrm{LDH}] = 100 \ \mathrm{X} \left\{ \frac{\left[\dfrac{[\mathrm{LDH}]_{\mathrm{assay}} - \left([\mathrm{LDH}]_{\mathrm{control\ (i)}} + [\mathrm{LDH}]_{\mathrm{control\ (ii)}}\right)}{[\mathrm{LDH}]_{\mathrm{killed\ epithelial\ cells}}} \right]}{\text{adhered yeasts cells}} \right\}$$

All experiments were performed in triplicate.

Analysis of *SAP* gene expression

RNA extraction

Prior to RNA extraction, the adhered yeasts were removed with 500 µl of lysis buffer (Invitrogen, USA) and transferred to Screw Cap Tubes (Bioplastics, NL). Then, glass beads (0.5 mm diameter, approximately 500 µl) were added and the tubes were homogenised twice for 30 s, using a Mini-BeadBeater-8 (Stratech Scientific, Soham, UK). After yeast cells disruption, the PureLink™ RNA Mini Kit (Invitrogen) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination the samples were treated with RNase-Free DNase I (Invitrogen).

Primers

The primers used for real-time PCR (RT-PCR) are described in Silva *et al.* [2] and their sequences are listed in Table 5.1.

Table 5.1: Primers used for real time-PCR analysis of *SAP* and control gene expression

Sequence (5'→ 3')	Primer	Target	PCR product size (bp)
GGAAGATCTGATGTGCCAACTACATTGA	Forward	*SAPT1*	1005
CGTGCGGCCGCTCTACAAAGCCGAGATGTCT	Reverse		
TTCTTCTAGTGGTACCTGGGTCAAAG	Forward	*SAPT2*	762
CATAGATCTCTAAACAATAGTGACATTAGA	Reverse		
ACTTGGATTTCCAGCGAAGA	Forward	*SAPT3*	165
AGCCCTTCCAATGCCTAAAT	Reverse		
GTACTCGAGCTCCTACAACTTCACCTCCT	Forward	*SAPT4*	1130
CATGGATCCCTATGTAAGTGGAAGTATGTT	Reverse		
GACCGAAGCTCCAATGAATC	Forward	*ACT1*	181
AATTGGGACAACGTGGGTAA	Reverse		

Synthesis of cDNA

To synthesize the complementary DNA (cDNA) the iScript™ cDNA Synthesis Kit (Biorad, USA) was used according to the manufacturer's instructions. For each sample 10 µl of extracted RNA was used.

Real-time PCR

Real-time PCR (CF X96™ Real-Time PCR System, Biorad, USA) was used to determine the relative levels of *SAPT1–4* mRNA transcripts with Actin 1 (*ACT1)* as a reference housekeeping gene. Each reaction mixture consisted on: working concentration of SsoFast™ EvaGreen® Supermix (Biorad, USA), 300 nM forward and reverse primer, and 1 µl of cDNA, in a final reaction volume of 20 µl. Negative controls (water) were included in each run. The relative quantification of *SAPT1-4* gene expression was performed by the ΔC_T method. Each reaction was performed in triplicate and mean values of relative expression were analysed for each *SAP* gene.

Statistical analysis

The results obtained were analysed using the SPSS 18 (Statistical Package for the Social Sciences) program. One-way ANOVA with the Bonferroni test was used to compare the number of adherent yeasts to epithelial cells, and cell activity and damage. All tests were performed with a confidence level of 95%. All the experiments were performed in triplicate and in three independent assays.

RESULTS

Cellular activity and the degree of cell damage as a result of the interaction of *C. tropicalis* with different human epithelial cell lines (TCC-SUP, HeLa, and Caco-2) as well as *SAPT1-4* gene expression are presented in Table 5.2 and 5.3. In general, *C. tropicalis* strains were able to adhere to the different epithelia and to cause a certain degree of cell damage and activity reduction. Moreover *SAP* genes were also expressed during epithelium colonization. However, these parameters were dependent on the yeast strain and on the epithelial cell line.

Table 5.2: Percentage of human cells activity inhibition and damage evaluated by MTS and LDH, respectively after *Candida tropicalis* adhesion to three different cells line. The *P* value obtained from the comparison between the two strains is also presented

Cell line	% cell activity Inhibition (±SD)			% cell damage (±SD)		
	U69 isolate	reference strain	*P* value	U69 isolate	reference strain	*P* value
TCC-SUP	17.96 (±3.90)	6.05 (±1.32)	*0.00	†14.24 (±3.73)	11.07 (±2.17)	0.35
HeLa	†4.81 (±0.91)	5.12 (±0.99)	1.00	†1.56 (±0.54)	†2.39 (±0.59)	1.00
Caco-2	†31.53 (±3.91)	†50.27 (±2.60)	*0.00	6.16 (±0.99)	†13.79 (±0.50)	*0.00

All values are means ± Standard deviations.
† Statistically different comparing among cell lines but, with the same strain ($P < 0.05$).
* Statistically different comparing between strains but, with the same cell lines ($P < 0.05$).

Curiously in Figure 5.1, strain U69, which is a clinical isolate from urine, adhered in significantly higher number (2.45 x 10^6 yeast/cm^2) to intestinal cells (Caco-2) than to urinary cells (TCC-SUP). In addition, this isolate induced a greater reduction in the cellular activity of intestinal cells than in

the urinary cells and presented highest levels of *SAPT1-3* expression (0.04; 0.03; 6.52, respectively). However, when in contact with urinary cells, strain U69 induced a greater percentage of cell damage (14.24%) and a higher expression of *SAPT4* (0.11) than when in contact with the other cell lines. The interaction of this urinary isolate with cervical cells (HeLa) resulted in low cellular activity inhibition (4.81%) and cell damage (1.56%) and this strain expressed only *SAPT3.*

Regarding the reference strain (ATCC 750), it adhered to TCC-SUP cells (2.30 x 10^6 yeast/cm^2), HeLa cells (1.56 x 10^6 yeast/cm^2) and Caco-2 cells (1.48 x 10^6 yeast/cm^2) in a similar extent ($P > 0.05$). Concerning the cellular response caused by the reference strain, the highest inhibition of cellular activity occurred in CaCo-2 cells (50.27%), which showed a greater degree of cell damage (13.79%) and a higher expression of *SAPT3-4* genes (9.71 and 0.49, respectively) than when in contact with the other two cell lines. In addition, HeLa cells showed less cellular response to the reference strain but higher expression of *SAPT1-2* (0.06 and 0.05, respectively).

It is important to highlight that both *C. tropicalis* strains affected HeLa cells in lower extent, causing low inhibition of cell activity and cell damage. *SAPT3* was the gene that exhibited the highest level of expression.

Table 5.3: Detection of secreted aspartyl proteinase (*SAPT1-4*) gene expression associated with pathogenesis adhering *Candida tropicalis* cell line using quantitative real-time polymerase chain reaction

Cell line	Relative expression of *SAP* genes*							
	U69 isolate				reference strain			
	SAP1	*SAP2*	*SAP3*	*SAP4*	*SAP1*	*SAP2*	*SAP3*	*SAP4*
TCC-SUP	0.02±0.01	0.01±0.00	5.01±0.63	0.11±0.05	0.04±0.00	0.01±0.00	0.77±0.85	0.07±0.03
HeLa	ND	ND	0.09±0.00	ND	0.06±0.04	0.05±0.00	5.91±0.37	0.08±0.01
Caco-2	0.04±0.00	0.03±0.01	6.52±0.02	0.05±0.01	0.05±0.02	0.02±0.00	9.71±0.01	0.49±0.00

* Mean arbitrary messenger RNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective ACT1 transcript level.
ND indicates that no gene expression was detected.

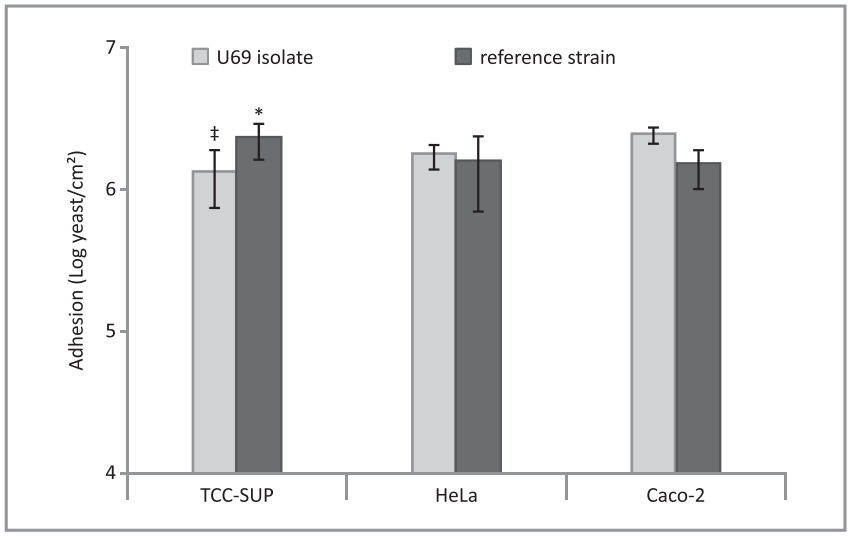

Figure 5.1: *Candida tropicalis* adhesion to the different cells line (TCC-SUP, HeLa, and Caco-2 cell lines), analysed by CV staining method. *Isolates with statistically different extent of adhesion ($P < 0.05$). ‡*C. tropicalis* adhesion to cell line statistically different from other cells line ($P < 0.05$). Error bars represent standard deviation.

DISCUSSION

Candida tropicalis is an opportunistic human pathogen, which colonizes several anatomically sites including skin, gastrointestinal and genitourinary tracts [13, 14]. Moreover, colonization by *C. tropicalis,* especially from specific body sites such as gastrointestinal and urinary tract, is related with a high risk factor for development of infection [11]. Several virulence factors seem to be responsible for *C. tropicalis* infections, which present high potential for dissemination, invasion and lethality [4, 15], namely the ability to adhere to human cells and to secrete enzymes such as proteases [1, 3, 16].

Several studies have been performed to clarify the behaviour of *Candida* species in the adhesion process, colonization and infection using human cell lines as a study model for *Candida* pathogenesis *ex vivo* [3, 9, 17-19]. However, comparatively to *C. albicans*, only few investigations have been performed to assess the virulence of *C. tropicalis*, particularly in the presence of different human cell lines. Thus, the major goal of the present

study was to investigate, *ex vivo,* the potential of *C. tropicalis* to colonize and damage urinary, vaginal and intestinal epithelium. Therefore, three different cell lines were used, namely: one from human bladder (TCC-SUP); other from human cervical carcinoma (HeLa); and the last from human colorectal adenocarcinoma (Caco-2). These cell lines are very often used to study, *in vitro*, mechanisms of interaction between *Candida* and gastrointestinal and genitourinary tracts [3, 8, 9, 12].

In the present study and corroborating other authors, *C. tropicalis* were able to adhere and to damage all the different epithelial cell lines used [8, 12, 18]. Furthermore *C. tropicalis* expressed a range of profiles of *SAP* genes [1, 2], although in a strain and cell dependent manner [3, 4]. In fact, *C. tropicalis* strains have shown different behaviours in their ability to invade human tissue with dramatic histopathological tissue alteration [2, 4, 18, 20].

Comparing with other studies [2, 3, 19], it is possible to verify that *Candida* species do not adhere in the same extent to the different mucosal type of cells, and also that there is not a strong correlation between the adherence ability to epithelial cells when the site of yeast isolation corresponds to a similar type of epithelium. It is important to highlight that both strains used in this study, U69 and the reference strain, are different clinical specimens and showed different infectivity. The strain U69 is a clinical isolate from a urine sample of a female patient from an oncology unit and the reference strain, ATCC 750, is a clinical isolate from a patient with bronchomycosis. Interestingly, the strain U69 adhered in higher extent to Caco-2 cells than to the other human cells and the reference strain adhered in similar extents to the different cell lines; curiously in higher number to TCC-SUP than U69 (Figure 5.1).

According to Sohn *et al*. [9] the interaction of *C. albicans* with Caco-2 cells is distinct from the interaction with epidermoid vulvo-vaginal cell line (A-431) and these authors suggested that this fact can be related with epithelial cells morphology and molecular events during adhesion. Furthermore, in a previous study with the same strains [3], it was

confirmed that *C. tropicalis* isolate U69 adhered to TCC-SUP cell line in a lower number than the reference strain.

Subsequent to the evaluation of *C. tropicalis* adhesion, it was studied the cellular response to this stimuli and interestingly both cell activity and integrity were affected by the yeasts. Although there is no direct correlation between inhibition of cell activity and cell damage, it was noticed (Table 5.2) that when cell inhibition is significantly high, cell damage is also notorious. To the authors' knowledge the related articles only evaluate cell damage (by LDH measurement) or cell activity inhibition but never both approaches. [17], showed that *C. famata* caused a low inhibition of cell activity after two hours of contact in opposition to *C. tropicalis* strains. In fact, opportunistic pathogenic yeasts, such as *C. tropicalis*, are able to promote cell immune response and progressive cell damage during infection [2, 4, 18]. Moreover, Silva *et al.* [2] demonstrated that *C. tropicalis* strains were able to cause significant tissue damage, also measured by LDH released, after long periods of yeast contact with cells (12 – 24h). Unfortunately, there are few studies regarding the effect of *Candida* species in human cells activity. Therefore, the present study is a step forward for the understanding of *C. tropicalis* pathogenesis.

The lowest damage was observed in HeLa cells comparatively to Caco-2 and TCC-SUP cells, highlighting that cell damage is dependent on the cell line type. According to Sohn *et al.* [9] *C. albicans* adhesion to epidermoid vulvo-vaginal cells seemed to occur slower when compared to the rate of adhesion to Caco- 2 cells and hyphal penetration into the cell lines monolayer started after 4 h leading to damage of the cellular substrate and marking the beginning of the tissue invasion phase in Caco-2 cells. Host cells injury by *Candida* has been described as a complex mechanism of interaction between yeasts and host cells [2, 9, 21]. However, this fact is mainly related with the time of infection, morphogenesis of *Candida*, morphology of host cells, and production of hydrolytic enzymes.

Concerning the human cells response and *SPAT* expression, it is worth observing that *C. tropicalis* adhesion affected both factors. During the

process of adhesion and invasion of host tissues, *Candida* species are known to secrete hydrolytic enzymes that cause damage on host cells membrane integrity, leading to dysfunction or disruption of host structures [1]. Furthermore, the expression of *SAP* genes by *C. tropicalis* has also been demonstrated during penetration of tissues and, evading macrophages after phagocytosis of yeast cells [1, 22, 23].

According to the results obtained in this study (Table 5.2), Caco-2 cells were the ones displaying the highest inhibition of activity when in contact with each of the tested strains and, in this situation, strain U69 and the reference strain expressed the highest levels of *SAPT1-3* and *SAPT3-4*, respectively (Table 5.3). However, strain U69 in contact with TCC-SUP cells, promoted a greater percentage of cell damage and a higher expression of *SAPT4* and the same happened with the reference strain when in contact with Caco-2 cells. Studies with *C. albicans* indicate that each *SAP* gene may be related to the yeast form and to a specific function [1, 2, 22, 24, 25].

Recent data for *C. albicans* indicated that *SAP1* to *SAP3* family genes are expressed by yeast cell only and contribute to the adhesion to human cells and tissue damage, whereas *C. albicans SAP4* expression is confined to hyphae, and have been further implicated with systemic infection and in the evasion to phagocytosis [25].

Furthermore, *SAPT3* transcript presented the highest level of gene expression for both strains. Silva *et al.* [2] studied the expression profiles of *SAP* genes for seven *C. tropicalis* strains in contact with reconstituted human oral epithelium and also determined the expression of the 4 genes but *SAPT2* and *SAPT4* transcripts were detected in a similar extent to *SAPT3*.

This fact can be related with the epithelium type and also with the different *C. tropicalis* strains studied. In the present case, the *SAP* gene expression was strain and human cell line dependent. It is important to highlight that there are only few studies [1, 2] reporting *C. tropicalis SAPT* gene expression during the adhesion to human cells and there is also

limited knowledge about the role of these enzymes in *C. tropicalis* adhesion and tissue damage.

In summary, the present study shows that *C. tropicalis* is able to adhere to different human cells influencing their response in a way dependent on cell type and yeast strain. This study also stresses out the importance of using more than one methodology to assess cell injury caused by *Candida* species. Moreover, cell damage and activity caused by *C. tropicalis* seem to be related with the expression of different *SAP* genes. As a preliminary study, this work only explored a small period of contact between yeasts and epithelial cells, therefore, more studies, including higher contact times could be of major interest as well as the inclusion of different clinical isolates. The knowledge of human cells response to *Candida* stimuli could further help in the development of new therapeutic strategies.

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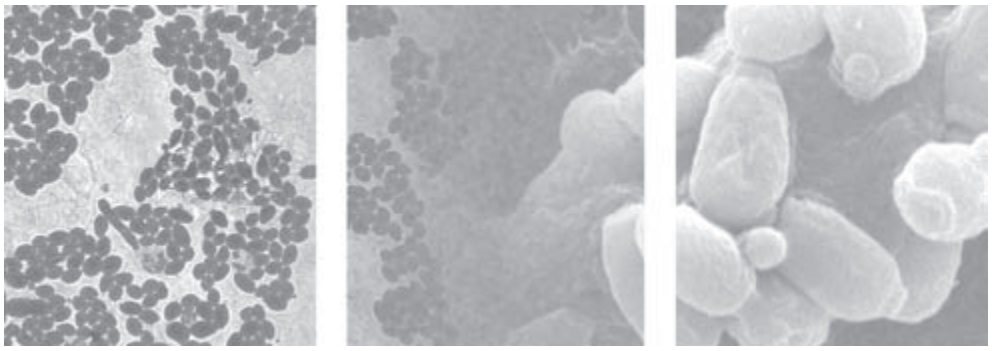
CHAPTER 6

Candida tropicalis biofilms: effect on urinary epithelial cells



ABSTRACT

It was to study the behaviour of *C. tropicalis* in biofilms of different ages (24 - 120 h) formed in artificial urine (AU) and their effect in human urinary bladder cells (TCC-SUP). The reference strain ATCC 750 and two isolates from urine (U69 and U75) were used in this study. The adhesion to TCC-SUP was evaluated using the crystal violet staining method and the TCC-SUP response was evaluated in terms of activity inhibition and cell damage. *Candida tropicalis* aspartyl proteinase (*SAPT*) gene expression was determined by real-time PCR. All strains of *C. tropicalis* were able to form biofilms in AU, although with differences among strains. *Candida tropicalis* biofilm cells were able to adhere to TCC-SUP, in general, independently of biofilm age. Yeasts affected TCC-SUP, with 120 h-biofilm cells of U69 and ATCC 750 strains causing the highest levels of cell injury. Generally, *SAPT3* was highly expressed and *SAPT4* was only detected in the reference strain. Overall, it is important to highlight that *C. tropicalis* cells detached from biofilms are able to colonize human cells and cause some injury and reduction of metabolic activity.

Keywords: *Candida tropicalis*; biofilm; artificial urine; adhesion; epithelial cells; *SAP* gene

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INTRODUCTION

Candida tropicalis ranks between the second or third non-*Candida albicans Candida* species (NCAC) most frequently isolated from patients with *Candida* infections [1-4]. According to epidemiological data, *C. tropicalis* has been related with urinary tract infections and haematological malignancy [2, 4-6]. Furthermore, the most important causes of *C. tropicalis* candiduria are associated with antibiotic therapy and indwelling catheterization [7-9].

Several virulence factors seem to be responsible for *C. tropicalis* infections, which present higher potential for dissemination and mortality in patients admitted in intensive care units (ICUs) than *C. albicans* or any other NCAC species [9]. Included in these virulence factors are: their ability to adhere and to form biofilms onto different indwelling medical devices; their capacity to adhere, invade and damage host human tissues due to enzymes production such as proteinases [9-12].

It is known that *Candida* biofilms have strategies to be more resistant to antifungal agents and virulent [13, 14] than the corresponding planktonic cells. Moreover, recent studies showed the capacity of *Candida* cells or biofilm parts to detach from a biofilm and to colonize distal sites, being able to disperse into the host environment and adhere to endothelial or epithelial cells and initiating an infection [15]. However, there is little knowledge about the effect of *C. tropicalis* biofilms on epithelial cells. Thus, the aim of this work was to study the behaviour of *C. tropicalis* biofilms, formed in the presence of artificial urine, and their effect in bladder cells.

MATERIAL AND METHODS

Candida tropicalis and growth conditions

Three strains of *C. tropicalis* were used in this study, one reference strain from the American Type Culture Collection (ATCC 750) and two clinical isolates (U69 and U75) obtained from patients with candiduria admitted to the intensive care unit and belonging to the archive collection of the University Hospital in Maringá, Paraná, Brazil. The strains were kept frozen at -80 °C in Sabouraud dextrose broth (SDB; Liofilchem, Italy) containing 5% (v/v) glycerol. For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h at 37 °C. Yeast cells were then inoculated in Sabouraud dextrose broth (SDB; Merck) and incubated for 18 h at 37 °C under agitation in an orbital shaker (120 rev/min). After incubation, yeast cells were harvested by centrifugation at 8000 × g for 5 min at 4 °C and washed twice with Phosphate Buffer Solution (PBS; pH 7.5; 0.01 mol/l). The remaining pellets were suspended in artificial urine (AU) and the cellular density adjusted to $1x10^7$ yeasts/ml, using a Neubauer chamber. Artificial urine (pH 5.8) was prepared according to Silva *et al.* [11].

Candida tropicalis biofilms formation

Biofilms, with different ages of maturation (24, 48, 72, 96 and 120 h), were formed on silicone coupons (1 x 1 cm^2) according to Silva *et al.* [11]. The coupons were placed in 24 well microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) and 1 ml of standardized *C. tropicalis* suspension ($1×10^7$ yeasts/ml in AU) was added to each well. The microtiter plates were incubated for 24-120 h at 37 °C in an orbital shaker (120 rev/min). Every 24 h, an aliquot of 500 µl of AU was removed and an equal volume of fresh AU added to each well. The silicone coupons used as controls were similarly treated but in the absence of *C. tropicali*s. After the defined times of incubation, the medium was aspirated and non-adherent *C. tropicalis* cells were removed by washing the silicone coupons with PBS.

Candida tropicalis biofilm characterization

Biofilms, recovered at each time point, were evaluated in terms of: (i) number of cultivable yeasts by colony formation units (CFU) enumeration; (ii) total biofilm biomass using the crystal violet staining method (CV); (iii) metabolic activity by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay.

Number of cultivable yeasts

The number of cultivable yeasts was determined by CFU enumeration, according to Silva *et al.* [11] with some modifications. Briefly, after biofilm formation and washing (as described previously), 1 ml of PBS was added to the silicone coupons and the biofilms were removed with a cell scraper (Orange Scientific, Belgium). The coupons, immersed in PBS, were sonicated (Ultrasonic Processor; Cole-Parmer) for 45 s at 30 W (parameters optimized to avoid cell lysis). The suspensions obtained were vortexed vigorously for 5 min and then serial decimal dilutions (in PBS) were plated onto SDA. Agar plates were incubated for 24 h at 37 °C, followed by CFU enumeration, and the results were recorded as CFU per unit area of coupon (CFU/cm^2). At the end, the coupons were stained using the CV assay to confirm the complete removal of biofilm [11]. Experiments were repeated on three occasions with individual samples evaluated in triplicate.

Biofilm biomass quantification by crystal violet staining

Biofilm formation was assessed by total biomass quantification using CV staining. Thus, at the defined time points of incubation and after the removal of non-adherent *C. tropicalis* by washing with PBS, the biofilms were stained in accordance to Silva *et al.* [11]. The final absorbance values were standardized according to the area of silicone coupons (Abs/cm^2). Experiments were performed in triplicate and repeated in three to five independent assays.

In situ biofilm metabolic activity

After biofilm formation (as described previously), the reduction assay of the tetrazolium salt 2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT; Sigma–Aldrich, USA) [16] was used to determine the *in situ* biofilm mitochondrial activity of *C. tropicalis* cells in the biofilms. The absorbance values were standardized per unit area of well (absorbance/cm^2).

Human urinary bladder epithelial cell line

The cell line TCC-SUP, derived from human urinary bladder epithelial cells (DSMZ - German Collection of Microorganisms and Cell Cultures) was used as model for analysing the colonization by *C. tropicalis* cells when in contact with their biofilms. Cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (D-MEM; Gibco, USA) containing 10% of fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (P/S; Gibco, USA). After achieving 80% of confluence, cells were detached using a 25% trypsin-EDTA (Gibco, USA) solution and cell concentration was adjusted to $1x10^6$ cells/ml with fresh D-MEM without P/S and added to a 24-well plate. Prior to the adhesion assays, the wells containing human cells monolayers were washed two times with PBS.

***Candida tropicalis* biofilms in contact with TCC-SUP cells**

The silicone coupons containing biofilms were removed from the wells, carefully inverted and immediately put in contact with the pre-formed monolayer of TCC-SUP cells and 1 ml of D-MEM without P/S was added to each well. After 2 h of contact at 37 °C under 5% CO_2, the coupons containing the biofilms were removed and each well washed once with PBS. The remaining yeasts attached to the monolayer were quantified using the CV staining method, according to Negri *et al*. [17]. The mean absorbance of yeasts was expressed as the absorbance per area of each well and standardized by the number of adhered yeasts per area of each well using *C. tropicalis* standard curve [17, 18]. All the procedures were repeated in triplicate in at least three separate assays.

Effect of *Candida tropicalis* on TCC-SUP cells

Determination of epithelial cells damage and activity assay

The effect of *C. tropicalis* on TCC-SUP cells was determined according to Negri *et al*. [19]. The release of lactate dehydrogenase (LDH) by epithelial cells into the culture medium was used as a measure of cell damage. The LDH concentration in the medium was measured after 2 h of *C. tropicalis* biofilm contact with human cells, using the CytoTox-ONE^{TM} kit (Promega, Madison, USA) following the manufacturer's instructions.

After the removal of the coupons and the washing step with PBS the epithelial cells activity inhibition was determined using the CellTiter 96® assay ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H–tetrazolium], MTS; Promega, USA). All the procedures were repeated in triplicate in at least three separate assays.

Analysis of *SAP* gene expression

SAP gene expression was evaluated for planktonic and biofilm *Candida* cells and also for adhered yeast cells to the human epithelial TCC-SUP cells. For planktonic cells, a suspension of *C. tropicalis* was adjusted to 1 x 10^7 yeasts/ml in AU was incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev/min). Finally, the yeast cells were harvested by centrifugation at 8000 × g for 5 min at 4 °C and the pelletized cells were suspended in 500 µl of lysis buffer (Invitrogen, USA). After biofilm formation on silicone, as described above, the coupons were rinsed with PBS once to remove the non-adherent cells and then the biofilms were scraped from the coupons into 500 µl of lyses buffer. Prior to RNA extraction, *C. tropicalis* cells attached to TCC-SUP cells were also scrapped from the 24-well plate into 500 µl of lysis buffer.

RNA extraction

Candida tropicalis samples were transferred to screw cap tubes (Bioplastics, NL), then, glass beads (0.5 mm diameter, approximately 500 µl) were added and the tubes were homogenised twice for 30 s, using a Mini-BeadBeater-8 (Stratech Scientific, Soham, UK). After yeast cells disruption, the PureLink™ RNA Mini Kit (Invitrogen) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination the samples were treated with RNase-Free DNase I (Invitrogen).

Primers, synthesis of cDNA and real-time PCR

The primers used for real-time PCR (RT-PCR), synthesis of cDNA and real-time PCR are described in Silva *et al.* [10].

Statistical Analysis

The results obtained were analysed using the SPSS 18 (Statistical Package for the Social Sciences) program. One-way ANOVA with the Bonferroni test was used in these tests. All tests were performed with a confidence level of 95%. All the experiments were performed in triplicate and in three independent assays.

RESULTS

***Candida tropicalis* biofilms characterization**

Number of cultivable yeasts

Figure 6.1 (a) presents the number of cultivable cells retrieved from biofilms formed on silicone coupons and this confirms that all *C. tropicalis* strains were able to form biofilms in the presence of artificial urine (AU). Furthermore, the number of cultivable yeasts from U75 and ATCC 750

biofilms were similar in all time points assayed. However, the clinical isolate U69 showed significantly less (P = 0.01) cultivable yeasts (1.60 x 10^5 CFU/cm^2) for the 24 h old biofilm and higher number of cultivable yeasts (1.08 x 10^7 CFU/cm^2) for the 48 h-biofilm, than the other two strains. In general, *C. tropicalis* biofilms showed a decrease in terms of the number of cultivable cells from 48 h to 72 h (significant differences for strains U69 and U75, $P < 0.05$).

Biofilm biomass quantification

Concerning, *C. tropicalis* biofilm biomass it is possible to observe (Figure 6.1 b) that there were some differences among the strains and among biofilms of different ages. The isolate U69 presented the highest biofilm biomass (P = 0.01) at 24 and 48 h, when compared with the other two strains. However, for 72 h biofilms the highest biomass (P = 0.01) was attained by strain U75. It is interesting to notice that in terms of total biofilm biomass the behaviour of the three different strains is completely different. While biofilm biomass of U75 and ATCC 750 varied along the time no variation was detected, for strain U69. In fact, strain U75 biofilm biomass presented a boost after 48 h but stabilizing after 72 h while the reference strain biomass increased significantly ($P < 0.05$) from 48 to 72 h and from 96 to 120 h, but decreased significantly ($P < 0.05$) from 72 to 96h.

In situ biofilm metabolic activity

The analysis of *in situ* biofilm metabolic activity (Figure 6.1 c) indicated that, although there were some differences in the first time points, there was a pattern of activity among the different strains after 72 h, namely, there was a significant increase ($P < 0.05$) from 72 to 96 h and a decrease from 96 to 120 h. Until 72 h, the different strains presented distinct behaviours, while *C. tropicalis* reference strain and U69 presented a decrease of activity from 24 until 72 h, strain U75 presented a slight increase from 24 to 48 h and a decrease from 48 to 72 h.

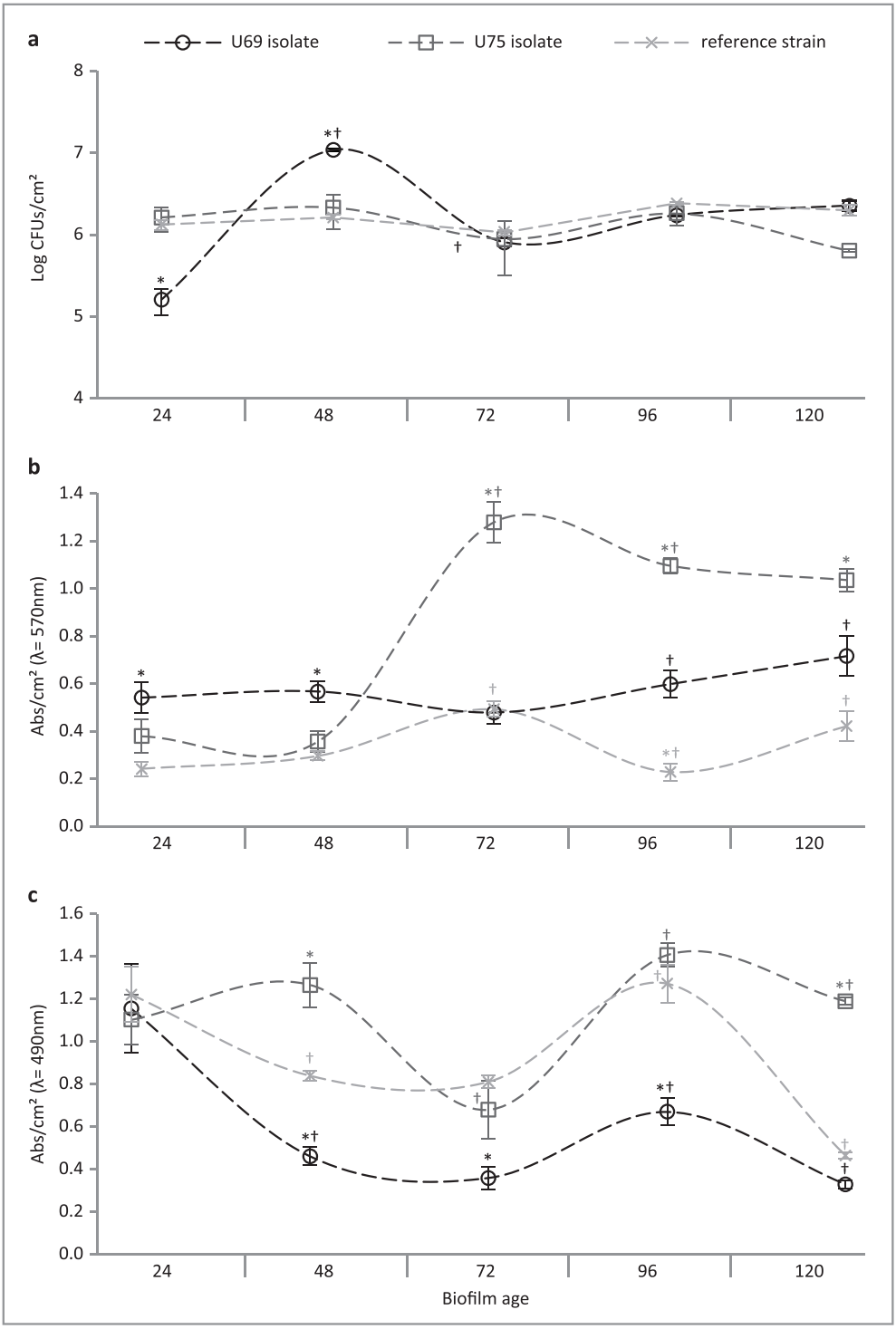

Figure 6.1: *Candida tropicalis* biofilms characterization. a) Number of cultivable yeasts by colony formation united; b) Biofilm biomass quantification by crystal violet; c) In situ biofilm metabolic activity by XTT. * Statistical difference among strains ($P < 0.05$); † Statistical differences among biofilms time point from the before biofilms time point ($P < 0.05$).

Candida tropicalis biofilms in contact with TCC-SUP cells

Number of adhered yeasts to epithelial cells

All *C. tropicalis* biofilm yeast cells were able to adhere to TCC-SUP cells independently of the biofilm age (Figure 6.2). Nevertheless, there were slightly differences in *C. tropicalis* adhesion ability to TCC-SUP cells, namely, the highest number of attached yeast cells was obtained for the 72 h-biofilm from isolate U75 and the lowest occurred for the 120 h-biofilm from isolate U69 ($P < 0.05$). The latter strain (U69) showed a higher variation in the profile of adhesion extent compared to the other two strains.

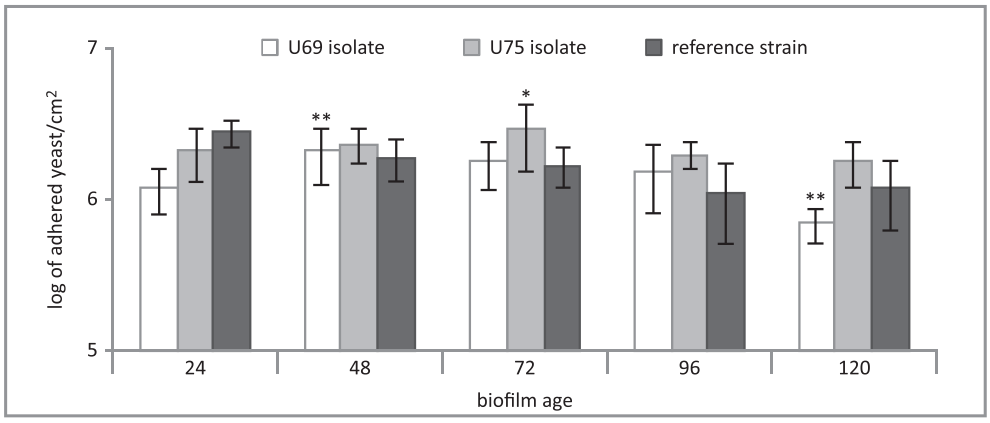

Figure 6.2: Number of *Candida tropicalis* from different biofilms time point attachment to human epithelial cells. * Statistical difference among strains ($P < 0.05$); ** Statistical differences from 24 to 48 h and 96 h to 120 h of U69 isolate biofilms time point ($P < 0.05$).

Effect of *C. tropicalis* on TCC-SUP cells

Determination of epithelial cells activity and damage

In general, *C. tropicalis* biofilm cells affected TCC-SUP cells after two hours of contact, although in a low extent (Table 6.1), and in a similar way for the three strains. It is important to highlight that TCC-SUP cells exhibited the highest ($P < 0.05$) percentage of damage after contact with 96 h-biofilm U75 cells and with 120 h-biofilms from strains U69 and ATCC 750. Overall, no direct correlation was observed between the profile of TCC-SUP cells

damage and activity inhibition caused by the different *C. tropicalis* biofilms. For instance, the human cells response induced by 72 h-biofilms is contradictory once there was a decrease in cell activity inhibition, but an increase in cell damage (with $P < 0.05$). However, it is interesting to notice that when human cells injury caused by biofilms (120 h-biofilm of strain U69) was more notorious, both tests were concordant, $P < 0.05$, (50.0% and 27.7 % of cell damage and inhibition of cellular activity, respectively).

Table 6.1: Percentage of cell activity inhibition and damage, evaluated by MTS and LDH, respectively, after *Candida tropicalis* adhesion to human TCC-SUP cell line. *P* values obtained from the comparison among the three strains and among biofilms with different ages (24, 48, 72, 96 and 120 h) are also presented

Reaction	Time (h)	Isolate		
		U69	U75	Reference
% activity inhibition (MTS)	24	16.0±2.4	11.0±1.6	5.2±1.6*
	48	16.6±2.4	15.9±3.6†	19.5±4.4†
	72	8.7±2.4†	4.5±1.1†	5.9±1.6†
	96	9.6±0.9	8.7±1.4	10.4±2.8
	120	27.7±3.4*†	7.7±1.6	6.1±0.8
% cell damage (LDH)	24	9.0±2.6	14.8±4.1	8.3±1.6
	48	12.3±2.6	14.1±3.5	14.8±1.0
	72	25.4±4.6†	18.4±3.5†	18.8±2.9†
	96	7.4±1.1†	26.6±4.9*†	12.4±2.3
	120	50.0±10.5†	20.6±1.3*	40.3±5.0†

Symbols represent statistical differences ($P < 0.05$) among strains (*) and biofilm's age (†). All values are means ± Standard deviations.

Candida tropicalis SAP gene expression

Analysing *C. tropicalis SAP* gene expression (Table 6.2), it can be noticed that *C. tropicalis* suspended cells grown in AU were not able to express *SAPT1* gene, even after having contacted with TCC-SUP cells. However, when grown in the sessile form, strains U69 and ATCC 750 from 48 h-biofilms were able to express *SAPT1* and this expression was preserved in cells from these biofilms that adhered to TCC-SUP cells. As regards *SAPT2* gene expression, although low, it was always expressed by *C. tropicalis* 96 h-biofilm cells but absent in the yeast cells adhered to TCC-SUP cells from these biofilms. Additionally, for U69 and ATCC 750 strains, *SAPT2* was

detected in 48h-biofilms and was also expressed by these cells in adhered to TCC-SUP cells, but in slight relatively lower amount.

Table 6.2: Detection by quantitative real-time polymerase chain reaction of secreted aspartyl proteinases (*SAPT1-4*) gene expression by planktonic and biofilm cells of *Candida tropicalis* and when adhered to the human epithelial TCC-SUP cell line

Strain	*Candida tropicalis* form	Relative expression of *SAP* genes*							
		C. tropicalis before contact with TCC-SUP				*C. tropicalis* adhered to TCC-SUP			
		SAP1	*SAP2*	*SAP3*	*SAP4*	*SAP1*	*SAP2*	*SAP3*	*SAP4*
U69 isolate	Planktonic	ND	ND	11.83±0.15	ND	ND	0.01±0.00	2.24±0.32	ND
	Biofilm 24 h	ND	ND	0.06±0.00	ND	ND	ND	0.24±0.16	ND
	Biofilm 48 h	0.02±0.00	0.02±0.03	0.30±0.00	ND	0.01±0.00	0.01±0.00	2.38±0.15	ND
	Biofilm 72 h	ND	ND	0.01±0.00	ND	ND	0.02±0.00	0.87±0.06	ND
	Biofilm 96 h	ND	0.01±0.00	2.89±0.16	ND	ND	ND	0.10±0.03	ND
	Biofilm 120 h	ND	ND	0.23±0.02	ND	ND	ND	0.21±0.05	ND
U75 isolate	Planktonic	ND	ND	25.31±1.22	ND	ND	ND	0.17±0.05	ND
	Biofilm 24 h	ND	0.03±0.02	2.35±1.03	ND	ND	ND	0.12±0.09	ND
	Biofilm 48 h	ND	ND	0.63±0.10	ND	ND	ND	0.26±0.08	ND
	Biofilm 72 h	ND	0.01±0.00	1.09±0.06	ND	ND	ND	0.26±0.06	ND
	Biofilm 96 h	ND	0.01±0.00	2.16±1.24	ND	ND	ND	0.31±0.09	ND
	Biofilm 120 h	ND	ND	1.15±0.29	ND	ND	0.01±0.00	0.56±0.31	ND
ATCC 750 ref.	Planktonic	ND	0.01±0.00	9.74±1.03	ND	ND	ND	0.01±0.00	0.02±0.00
	Biofilm 24 h	ND	ND	0.54±0.23	ND	ND	0.01±0.00	0.18±0.05	3.04±0.00
	Biofilm 48 h	0.01±0.00	0.07±0.06	20.95±2.98	0.02±0.00	0.18±0.00	0.01±0.00	0.34±0.06	ND
	Biofilm 72 h	ND	ND	0.05±0.03	ND	ND	ND	0.40±0.01	ND
	Biofilm 96 h	ND	0.01±0.00	1.53±0.68	ND	ND	ND	0.49±0.14	ND
	Biofilm 120 h	ND	ND	ND	ND	ND	ND	0.21±0.16	ND

* Mean arbitrary messenger RNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective *ACT1* transcript level.
ND indicates that no gene expression was detected.

In opposition to the other *SAPT* genes, *SAPT3* was expressed and in the great majority of situations, except by ATCC 750 120 h-biofilm cells. Interestingly, the amount of this *SAP* gene expressed by planktonic cells is much higher than the amount expressed by biofilm cells but this capacity is lost when planktonic cells adhere to the epithelium. It is also possible to

observe that, in general, the expression of *SAPT3* was lower for *C. tropicalis* cells adhered to TCC-SUP cells issuing from biofilms. Interestingly, *SAPT4* was only expressed by the reference strain, and in few situations (48h-biofilms and adhered yeasts grown in suspension and from 24 h-biofilms).

DISCUSSION

Candida species are the second most common cause of medical device infections, especially in patients from ICUs, which are associated with the highest pathogen-specific mortality [20, 21]. Furthermore, *C. tropicalis* and other *Candida* species are able to form biofilms, and their presence during infection has been related to higher mortality rates compared with isolates which are not capable of forming biofilms [21, 22]. Moreover, recent studies have demonstrated the ability of *C. tropicalis* to form biofilms in the presence of AU, under static and dynamic conditions [11, 12, 23].

In accordance with other studies [11, 12, 23], the strains of *C. tropicalis* assayed herein were able to form biofilms in the presence of AU (Figure 6.1), although in a strain and time dependent way. Similarly, a study by Jain *et al*. [23] with *C. albicans, C. glabrata* and *C. tropicalis,* using AU and RPMI 1640, showed that biofilm formation varied considerably among isolates under both growth conditions.

Observing the biofilm profile along time (Figure 6.1), no consistent pattern can be noticed among the different strains. The only similarity among strains is an increase in the number of cultivable cells and biofilm metabolic activity from 72 to 96 h biofilms. Variations among *C. tropicalis* strains concerning biofilm formation are expected due to physiological differences among strains [18, 22, 24]. Furthermore, as reported before [16, 18, 22] *C. tropicalis* species mature biofilms consist of a dense and heterogeneous network of yeast, pseudohyphae and hyphae and these forms are not always similar among *C. tropicalis* strains. These results corroborate other studies reporting that biofilm kinetics is strain dependent [11, 24]. For instance, in the present situation, U69 strain 24 h-

biofilm presented the lowest number of cultivable yeasts (1.60 x 10^5 CFU/cm^2; *P* = 0.01), although, showing the highest biofilm biomass (0.54 Abs/cm^2; *P* = 0.01). However, in a previous work [18], using RPMI 1640 as growth medium, U69 biofilm biomass was similar to the other *C. tropicalis* strains, which highlights that biofilms are dependent on growth medium, carbohydrate supplementation and the nature of the colonized surface [11, 22-24]. Furthermore, it has been reported that cultivable yeast cells and biofilm metabolic activity seems to be dependent of biofilm maturity with an increase in those parameters along biofilm development [16, 24, 25]. The present results underline strain differences in terms of biofilm metabolic activity and number of cultivable cells, which could have important implications in terms of *C. tropicalis* relative virulence.

Moreover, it should be stressed that the extracellular matrix of biofilms also accounts for total biofilm biomass and smaller amounts of cultivable cells do not mean a less amount of extracellular matrix because this is a trait strain specific and dependent on environmental conditions [12, 22]. Therefore, these differences on *C. tropicalis* biofilms can explain the increased difficulty on the treatment of infections related to this species. Moreover, these findings may have high significance concerning its pathogenic potential when extrapolated to *in vivo* situations.

It is known that during the development of *Candida* biofilms, some dispersion/detachment of cells or dissolution of biofilm pieces can occur [12, 15]. This is an important phenomenon, since the dispersed and detached cells could be responsible for the subsequent establishment of disseminated candidiasis at distal organs [15, 26]. Furthermore, in a recent study with *C. albicans*, Uppuluri *et al*. [15], have demonstrated that dispersed cells display a distinct phenotype, which is associated with an increased virulence. Additionally, according to epidemiological data, *C. tropicalis* infection is strongly connected with the presence of biofilms in urinary catheters [21, 23, 27-29]. Thus, it is extremely important to study the ability of *C. tropicalis* cells detached from pre-formed biofilms to colonize human epithelial cells and the consequent degree of damage.

Consequently, biofilms with different degrees of maturation, were put in contact, for two hours, with human urinary bladder epithelial cells (TCC-SUP cells) in order to evaluate the effect of biofilm cells on this epithelium. Along with the determination of the number of adhered yeasts to TCC-SUP cells (by CFU quantification), human cells damage and activity inhibition were also assessed. The results are indicative that cells from all *C. tropicalis* biofilms (independently of their age) were able to detach and colonize TCC-SUP cells (Figure 6.2), although in a strain dependent way. Other studies have shown that *C. tropicalis* planktonic cells extent of adhesion to human cells is also strain specific [10, 18, 30].

It is interesting to highlight that biofilm yeast cells adhered to TCC-SUP cells in a similar extent of their planktonic counterparts [18]. Uppuluri *et al*. [15] reported a different behaviour for *C. albicans* adhesion to endothelial cells, since yeast cells dispersed from biofilms adhered in larger numbers than planktonic ones. Maybe, this fact can be related with differences between the two yeast species or the differences between the human cells used, which determine very specific interactions with yeast cells [31, 32]

Besides, the quantification of the number of yeasts adhered to TCC-SUP it is of major importance to understand *Candida* pathogenesis – their effect on human cells. Other studies are indicative that dispersed cells from yeast biofilms caused increased human cells damage when compared to planktonic yeast cells [15]. Moreover, human cells damage is reported to be dependent on the contact time and yeast infectivity rate [10, 15, 33]. Overall, the present results point out that older biofilms (96 h or 120 h) induced higher cell damage (Table 6.1) than younger ones, however in a strain dependent manner. Therefore, these data highlight the virulence potential of *C. tropicalis* cells dispersed from biofilms, since they seem to be a causative agent of an increased LDH release by TCC-SUP cells.

In fact, opportunistic yeast pathogens, such as *C. tropicalis*, are able to promote cell host immune response and progressive cell damage during infection [10, 30, 34]. However, in this study no direct correlation was observed between cell damage and inhibition of cellular activity, namely

adhered yeast cells from 72 h-biofilm, induced high damage but a low extent of inhibition of cell activity. Nevertheless, it is very interesting to notice that when the inhibition of cellular activity was significantly higher (for U69 120 h-biofilm) cell damage was also the highest. This underlines that these two factors are only in direct relation when the degree of injury is very high. The lack of a direct relation between cell activity inhibition and cell damage at low levels of injury can be explained by the fact that, during the first stage of human cells colonization by *Candida*, the cellular defence mechanisms are initiated, which may cause an increase of metabolic activity before the onset of any damage [34-37]. Moreover, the present results point out that despite no significant differences observed in terms of *C. tropicalis* strains ability to colonize the TCC-SUP cells a direct correlation was not observed among the specific biofilm characteristics and the injury caused to human cells.

It has been widely reported that, during the adhesion and invasion processes of host tissues, *Candida* species are able to secrete hydrolytic enzymes that cause damage on host cells membrane integrity, leading to dysfunction or disruption of host structures [38, 39]. *Candida* secreted aspartyl proteinases (Saps) have been associated with tissue invasion and their secretion is also associated with the inflammatory host response [40]. Nowadays, several studies have been performed with the aim to elucidate the role of each Sap on the development of the host infection process. Recent data indicate that Sap1 to Sap3 family are probably the principal *C. albicans* proteinases involved on mucocutaneous infections *in vivo* and, also on adhesion to human cells and tissue damage. Furthermore, *C. albicans* Sap4 to Sap6 family proteinases have been implicated with systemic but not mucosal infections and in the evasion of phagocytosis [39, 41-43]. Additionally, the expression of *SAP* genes by *C. tropicalis* (*SAPT*1 to *SAPT*4) has also been demonstrated on the surface of fungal elements penetrating tissues during disseminated infection and evading macrophages after yeast cells phagocytosis [10, 38, 44, 45]. Although, little is known about the contribution of *SAP* genes on *Candida* biofilm formation, recent findings showed that sessile *C. albicans* cells adhered to abiotic surfaces secrete more Saps than their planktonic counterparts [46].

Moreover, Nailis *et al*. [47] reported that *SAP* genes are upregulated in *C. albicans* biofilms grown in different model systems (biotic and abiotic) at different stages of maturity. Thus, with the aim to understand the role of Saps as causative agents of the injury caused to TCC-SUP cells, the levels of expression of *C. tropicalis SAP* genes were assessed for planktonic and biofilm cells before and after adhering to TCC-SUP human cells.

Real time PCR analysis (Table 6.2) revealed that *SAPT* gene expression by *C. tropicalis* grown in AU (in planktonic and biofilm form and colonizing TCC-SUP cells) showed, in general, a higher level for *SAPT3* expression followed by lower levels of *SAPT2*, *SAPT1* and *SAPT4.* These features were similar to those described by Silva *et al.* [10] who studied the expression profiles of *SAP* genes by seven *C. tropicalis* strains in contact with reconstituted human oral epithelium: all strains also expressed the 4 genes (*SAPT1 – 4*), moreover *SAPT2* and *SAPT4* transcripts were detected in a similar extent to *SAPT3* and the majority of strains did not express *SAPT1*. Similarly, in the present study, only two strains (U69 and ATCC 750 strains) in 48 h-biofilms were able to express *SAPT1,* but in planktonic form this gene expression was not detected. Furthermore, the high expression of *SAPT3* points out its importance in the pathogenesis of *C. tropicalis* strains under these conditions.

In order to confer maximum benefits for *Candida* pathogenicity *SAP* genes family are activated efficiently and in flexible way at specific time points during colonization and infection processes. Moreover, *SAP* genes expression is strongly correlated with environment where *Candida* is grown [39, 41, 44]. Nails *et al*. (2010) detected differences in *C. albicans SAP* genes expression between *in vitro* grown biofilms and *in vivo* model. Interestingly, *SAP1, SAP2, SAP4* and *SAP6* were highly upregulated in biofilms grown *in vitro* and the expression of *SAP2, SAP4* and *SAP6* was also high in the *in vivo* model. In the present study there is also a different gene expression among the different modes of growth. For instance, although *C. tropicalis* 96 h-biofilms exhibited *SAPT2* expression, when these biofilm cells colonize TCC-SUP cells this gene is not expressed. Curiously, *SAPT4* was only detected in ATCC 750 biofilms and at specific biofilm ages. Other

studies indicate that there is an optimum pH for *C. tropicalis*-secreted aspartic proteinases activity, therefore making *SAP* gene expression strain and epithelium dependent [10, 38, 44]. It is important to highlight that there are only few studies reporting *C. tropicalis SAPT* genes expression during the adhesion to human cells and there is also limited knowledge about the role of these enzymes in *C. tropicalis* biofilms. This could suggest that Saps do not play a significant role in the reduction of epithelial cells activity and damage caused by *C. tropicalis* strains.

In summary, the present study shows that *C. tropicalis* detached from biofilms are able to colonize human cells and cause some injury: damage and reduction of metabolic activity. Moreover, it is important to highlight that *C. tropicalis SAP* genes are expressed during *C. tropicalis* biofilm formation. Furthermore, to our knowledge, this is the first report of *C. tropicalis SAP* genes expression during biofilm formation and when biofilm cells colonize TCC-SUP cells, and the results imply a limited role of these enzymes in human cells damage and metabolic activity reduction in the conditions assayed. Nevertheless, *SAPT3* transcript presented the highest level of gene expression by *C. tropicalis* assayed, regardless of biofilm age. However, more studies have to be performed to clarify if these *C. tropicalis SAPs* genes are associated with biofilm development and *C. tropicalis* virulence potential.

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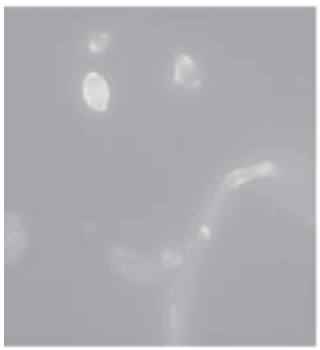
CHAPTER 7

Final conclusions
future perspectives



CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Candida tropicalis is a common yeast species related to nosocomial candidemia and candiduria. Several virulence factors seem to be responsible for *C. tropicalis* infections, which present high potential for dissemination and mortality. Adhesion to host surfaces (medical devices and host cells), as well as biofilm formation, are considered the first steps to initiate *Candida* infection. Hence, the colonization of indwelling devices like urinary catheters by *C. tropicalis* poses a critical problem.

In fact, several important studies have been describing the potential of *C. tropicalis* to form biofilms, although, most of the *in vitro* research do not always mimic what happens in reality. Thus, in Chapter 2, it is reported for the first time the formation of *C. tropicalis* biofilms in urinary catheters in a flow model, although using artificial urine (AU) to obtain more reproducible results. The model developed appears to be suitable for studies simulating the real conditions and furthermore it was possible to observe that *C. tropicalis* was able to adhere and to form biofilms along the entire length of both types of urinary catheters, latex and silicone.

Furthermore, all *C. tropicalis* strains assayed along this work were able to form biofilms independently of the culture medium used, RPMI 1640 (Chapter 4) or AU (Chapter 6). However, there were differences among strains when biofilms were formed in AU (Chapter 2 and 6). Curiously, although the isolate U69 adhered significantly in higher extent to both types of catheters than the reference strain (Chapter 2), U69 biofilms contained significantly less cultivable cells but higher biofilm biomass than those of the reference strain. A similar behaviour was observed for U69 24 h biofilms, formed in silicone coupons in AU (Chapter 6). In older biofilms, the clinical isolate U69 showed significantly higher number of cultivable yeasts (eg 48 h biofilm), than the other two strains (reference strain and U75 clinical isolate) assayed. Nevertheless, *C. tropicalis* biofilms grown in AU seem to stabilize in terms of number of cells over time (after 48 h) both in dynamic and static model.

The formation of a biofilm inside or in the outer surface of medical devices causes a multiplicity of problems. One of the most frequent consequences is the infection of distal sites. Biofilm associated infections are very difficult to treat, especially due to the conjunction with an increased occurrence of multi-drug resistance by *Candida* species. It is important to emphasise that one clinical isolate (U69) was resistant and other four (U16, U29, U75 and CL012) were susceptible-dose dependent to itraconazole (Chapter 4). Since, those strains were effective biofilm formers, there is an increased risk factor associated to those strains in the development of candidosis, especially when these strains are able to detach from biofilm and to colonize other surfaces, as host cells. In this work, under flow conditions, *C. tropicalis* strains were able to detach from catheters (latex and silicone) and to move upflow against the stream, demonstrating undoubtedly their capacity to colonize distal sites (Chapter 2).

Adhesion to and invasion of host cells by *Candida* species is considered the first step in systemic infections. Although *in vitro* studies of adhesion capability are essential to characterise the virulence of those *Candida* species, the assessment of adhesion by traditional methods is very time consuming. Therefore, an easy to perform and reproducible technique was developed to assess the adhesion ability of *Candida* species to epithelial cell lines (Chapter 3). The method was validated for the different *Candida* reference strains of different species by comparison with traditional microscope observation and enumeration. High correlation values (r^2= 0.9724–0.9997) between the number of adherent yeasts (microscope enumeration) and absorbance values were obtained for an inoculum concentration higher than 10^6 cells/ml. Additionally, it was also verified that all *Candida* species adhered in a greater extent to epithelial cells than to silicone. However, different *Candida* species have different sizes and absorb distinct amounts of dye, which does not allow comparison of the level of adhesion through direct CV absorbance readings. So, after standardization of the results using the corresponding equation for each species, *C. glabrata* (initial cell density: 10^8 yeast/ml), and *C. tropicalis* (initial cell density: 10^7 yeast/ml) adhered significantly more than other *Candida* species to epithelial cells. Although these results correspond to a

low number of strains, it is important to highlight these data since *C. tropicalis* is a common species related to nosocomial candidaemia and candiduria, and *C. glabrata* is now emerging as an important agent in both mucosal and bloodstream infections.

In this context, while investigating more about *C. tropicalis* virulence, with more samples (Chapter 4), it was possible to confirm that *C. tropicalis* strains adhered significantly in higher number to epithelium than to silicone when in RPMI 1640 culture medium. Interestingly, the same happened (Chapter 3) in a different culture medium (PBS), indicating that there is a greater predilection of *C. tropicalis* for urinary bladder cells (TCC-SUP) than silicone coupons, independently of culture medium.

Furthermore, regarding *C. tropicalis* infectivity (Chapter 5) it was shown that *C. tropicalis* strains were able to adhere to the different human cell lines, although, in a strain and cell line dependent manner. Strain U69, which is a clinical isolate from urine, adhered in significantly higher number to intestinal cells (Caco-2) than to urinary cells (TCC-SUP) and cervical cells (HeLa). Regarding the reference strain (ATCC 750), it adhered to TCC-SUP cells, HeLa cells and Caco-2 cells in a similar extent. Also, *C. tropicalis* biofilm cells (Chapter 6) were able to adhere to TCC-SUP cells, in general, independently of biofilm age, even so the highest extent of yeast attachment was obtained for the 72 h old U75 biofilm and the lowest occurred for the 120 h-biofilm from strain U69. It is important to note that there may be a specific interaction between each human cell type and each strain of *C. tropicalis.* Moreover*,* there is not a strong correlation between the adherence ability to epithelial cells when the site of yeast isolation corresponds to a similar type of epithelium.

Concerning the human cells response to *C. tropicalis* colonization, it was noticed that both planktonic (Chapter 5) and biofilms (Chapter 6) yeasts were able to affect the human cells. In general, the highest cell activity inhibition was observed for Caco-2, followed by TCC-SUP and HeLa cells (Chapter 5). The highest percentage of cell damage was observed for TCC-SUP in contact with the U69 isolate and for Caco-2 in contact with the

reference strain. Yeasts from biofilms (Chapter 6) affected TCC-SUP cells, with 120 h-biofilm cells of U69 and ATCC 750 strains causing the highest levels of cell injury (damage and inhibition of cell activity). Comparing the effect of dispersed cells from *C. tropicalis* biofilms (Chapter 6) with planktonic cells (Chapter 5), it was observed that the first caused more cellular damage than the latter. Therefore, these data highlight the virulence potential of *C. tropicalis* cells dispersed from biofilms, since they seem to be a causative agent of an increased damage, specially on TCC-SUP cells.

Although no direct correlation between inhibition of cell activity and cell damage was found, it was noticed that when cell inhibition is significantly high, cell damage is also notorious. Unfortunately, there are few studies regarding the effect of *Candida* species in human cells activity. Therefore, the present study is a step forward for the understanding of *C. tropicalis* pathogenesis.

During the adhesion and invasion processes of host tissues, *Candida* species are able to secrete hydrolytic enzymes that cause damage on host cells membrane integrity, leading to dysfunction or disruption of host structures. Regarding expression of enzymes (proteinases, phospholipase and haemolysins) on specific agar medium (Chapter 4), all *C. tropicalis* strains assayed were able to express total haemolytic activity. However, proteinases were only produced by four isolates (U16, U29, CL012 and B1). Moreover, only one *C. tropicalis* strain was phospholipase positive (CL012). According to these results, proteinase and phospholipase expression can vary according to strain and the site of isolation. Although the methods used to test the production of these enzymes are simple and fast, they are not excessively accurate, specially compared with molecular methods that can detect gene expression.

Thus, real-time PCR analysis (Chapter 5) was also used and it revealed a wide range of expression profiles of Secreted Aspartyl Proteinases (*SAP)* genes for both *C. tropicalis* strains (U69 and reference strain) in contact with the different types of epithelial cells. According to data obtained in

Chapter 5 and 6, *SAPT* genes may be involved on pathogenesis of *C. tropicalis*, affecting the human cell lines. Moreover, *C. tropicalis SAP* genes were expressed during *C. tropicalis* biofilm formation (Chapter 6). Furthermore, in both studies (Chapter 5 and 6), *SAPT3* produced by adhered and biofilm yeasts were highly expressed comparing with other *SAPT* genes. It is important to emphasize that human cells response to *C. tropicalis* adhesion, as well as *SAPs* production, is strain and epithelial cell line dependent. There is limited knowledge about the role of these enzymes in *C. tropicalis* adhesion and tissue damage. Hence, this is an important point that can contribute for the enhanced understand of *C. tropicalis* pathogenesis.

These findings lead to other questions, which would greater contribute to the understanding of the virulence factors used by *C. tropicalis* during the development of candidosis. Some of the suggestions that should be taken into consideration for future investigations are:

1. Assessment of mechanisms related with the phenomen of yeast cells detachment from *C. tropicalis* biofilms, investigating architecture, cells interaction and quorum sensing of *C. tropicalis* biofilms.
2. Characterization of the main virulence factors of *C. tropicalis* cells detached from biofilms and evaluation of their antifungal resistance.
3. Evaluation of the role of phospholipases and haemolysins in *C. tropicalis* pathogenesis by genomic approach, by analysing the levels of gene expression during the interaction with human cells.
4. Assessment of molecular interactions between human cells and *C. tropicalis* analysing cellular response and factors related with the process of adhesion and infectivity.
5. Summarising, these factors together with those previously exposed, may come together to help clarifying *C. tropicalis* behaviour during the process of infectivity and, furthermore, could contribute to develop new antifungal agents targeting these mechanism of *Candida* virulence.