Semana de Engenharia 2010
Guimarães, 11 a 15 de Outubro

INSIGHTS INTO *CANDIDA* WORLD: THE EXTRACELLULAR MILIEU

Margarida Martins, Mariana Henriques, Rosário Oliveira
Center of Biological Engineering
E-mail: margarida.martins@deb.uminho.pt; mcrh@deb.uminho.pt; roliveira@deb.uminho.pt

KEYWORDS

Candida species, extracellular DNA, extracellular alcohols

ABSTRACT

Over the last years *Candida* species, including *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis*, have emerged as significant pathogens. This work aimed at bringing insights into *Candida* species world by the analyses of compounds released into the extracellular medium namely extracellular DNA (eDNA) and alcohol compounds. Concerning eDNA, our results present evidence that this is a key element of the *C. albicans* biofilm extracellular matrix, contributing to biofilm integrity and antifungal resistance. With respect to extracellular alcohols, the results presented herein show that *Candida* species secrete a cohort of alcohols that are able to regulate their virulence traits (in vitro and in vivo). Taken together, these studies represent an important contribution to our understanding of the composition of the extracellular milieu of *Candida* species and its relationship with the regulation of *Candida* biology, opening the possibility of the development of new treatment and/ or diagnostic strategies to combat candidiasis.

INTRODUCTION

Starting in the early 1980s, fungal diseases began to constitute a major public health problem. The observed increase in opportunistic fungal infections has parallel with the expansion in populations with metabolic and immunosuppressive diseases, and mucosal or cutaneous barrier disruption, as a consequence of the advances in medical practices (Hazen, 1995;Pfaller and Diekema, 2007). Overall, *Candida* species are now recognized as major agents of opportunistic fungal infections worldwide. Although for many years *Candida* infections were synonymous of diseases caused by *Candida albicans*, the most recent surveillance studies indicate that Non-*Candida albicans Candida* (NCAC) species, particularly, *Candida dubliniensis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis* are emerging as pathogens (Lewis, 2009;Pfaller and Diekema, 2007;Loreto et al., 2010). This species distribution shift has been attributed to NCAC species intrinsic or acquired resistance to antifungal agents, namely fluconazole (Lewis, 2009). The recognition of *Candida* infections as a life-threatening problem associated with high mortality rates and costs, due to increased length of patients hospitalization, antifungals acquisition, ineffective treatment, and antifungals side effects (Hennen, 2009), probably are responsible for the increasingly interest in the study of *Candida* and candidiasis.

Candida species live in complex and dynamic environments, being consequently exposed to external cues. In fact, to colonize so diverse niches such as the skin, gastrointestinal tract, or oral cavity, the cells need to adapt, for example, to different pH and oxygen levels, and microbial flora. This plasticity may involve the sensing of multiple environmental inputs and adaptation/ response through the expression of virulence factors. *Candida* species virulence factors comprise the ability of *C. albicans* and *C. dubliniensis* to undergo a morphological transition between yeast and filaments (pseudohyphae and/or hyphae), the ability of *Candida* species to form biofilms, and to release molecules into the extracellular medium including proteins and alcohols such as *E*,*E*-farnesol (farnesol) (Calderone and Fonzi, 2001;Li et al., 2007;Haynes, 2001;Trofa et al., 2008;Sullivan et al., 2005;Samaranayake and Samaranayake, 1994;Langford et al., 2009). Therefore, one of the challenges in fundamental research is to identify the components that mediate these interactions and to understand their effect and strength on cell phenotype, to ultimately develop functional models that may help to get a better understanding on cellular biology and pathogenesis (Sauer et al., 2007).

In the last decade it was observed an increase in the "omics" approaches in the *Candida* field, crucial to the elucidation of the fungal biological and physiological responses to its environment. The genomic era started with *C. albicans* (Jones et al., 2004) and *C. glabrata* (Dujon et al., 2004) genomes sequence, but only recently *C. dubliniensis* (Jackson et al., 2009), *C. parapsilosis*, *C. tropicalis*, *Candida guilliermondii*, and *C. lusitaniae* (Butler et al., 2009), but not *C. krusei*

genome sequences were published. The initial genomic studies in *C. albicans* opened the possibility to perform transcriptomic studies that, by measuring the dynamic expression of messenger RNA (mRNA), have been used to identify genes relevant to physiological processes, as dimorphism (Nantel et al., 2002) or in particular niches, such as biofilms [reviewed by Coenye (2010)]. Moreover, the proteomic studies, through the analysis of global patterns of protein expression, have been applied to the identification of key proteins related for example with cell adhesion or drug resistance [reviewed by Thomas et al. (2006)]. Overall, the combination of these approaches has been helpful to get a better understanding on *C. albicans* existence as a human commensal and pathogen.

However, the cells adjust their metabolism, and consequently their metabolites, according to the different physiological states (Oldiges et al., 2007). The whole group of metabolites -the metabolome- link DNA, mRNA, proteins, complex interactions, and cellular pathways with environmental stimuli, thus being considered functional entities of the cells (Zhang et al., 2010;Jewett et al., 2006). Metabolomic -the quantification of the metabolome- or metabolic profiling -the quantification of a set of pre-defined metabolites belonging to a class of compounds or to members of particular pathways- (Oldiges et al., 2007) are unexploited topics in the *Candida* field. However, increased awareness is arising in the scientific community, with the "Biochemical Pathways" feature being recently added as a tool at the Candida Genome Database (CGD, http://www.candidagenome.org/) (Skrzypek et al., 2010).

Due to the diversity of fungal extracellular metabolites (Frisvad et al., 2007) and their dynamic regulation, the complete analysis of the metabolome is considered a difficult task (Villas-Boas et al., 2005). Even though, it is possible to perform target analysis for the detection and quantification of a single or a small set of metabolites (Oldiges et al., 2007), thus achieving partial insights into the metabolome.

Thus, this work aimed at bringing insights into *Candida* species world, through the evaluation of compounds released into the extracellular medium, namely extracellular DNA (eDNA) and alcohol compounds, and the elucidation of the interactions of the identified molecules with virulence traits of *Candida* species.

EXTRACELLULAR DNA

The interest on the study of *C. albicans* eDNA was raised by evidences showing that: (i) eDNA accumulates into the extracellular medium in vitro and in vivo (Kasai et al., 2006), (ii) eDNA can regulate the host immune response (Yordanov et al., 2005;Dimitrova et al., 2008;Miyazato et al., 2009), and (iii) deoxyribonuclease (DNase) reduces mature biofilm biomass (Al-Fattani and Douglas, 2006). Even though, this molecule has not received considerable attention in the *Candida* biofilm field, and it has not been addressed, for example, whether it is a component of biofilm extracellular matrix (ECM) or not, or further exploited regarding its contribution to biofilm lifestyle.

Using a fluorometric method, we were able to detect the accumulation of eDNA in the ECM extracted from *C. albicans* biofilms formed under conditions of flow, although the quantity of eDNA detected differed according to growth conditions, in particular with regards to the medium used to grow the biofilms (Table 1) (Martins et al., 2010b).

Table 1: *C. albicans* 48-h biofilm ECM eDNA and protein content

Growth medium	eDNA (ng)	Protein (mg)	eDNA/ protein (ng/mg)
RPMI	1518 ± 822	0.48 ± 0.2	3045.4 ± 227.3
YPD	180 ± 160	0.42 ± 0.1	339.6 ± 265.6
YNB	1.8 ± 1.6	0.10 ±0.1	13.0 ± 8.8

One of the points under debate in the *Candida* scientific community relates to the origin of *C. albicans* eDNA. Accumulated evidences in the bacterial field suggest that eDNA may be released through quorum sensing, by specific secretion mechanisms, and cell lysis (Draghi and Turner, 2006;Spoering and Gilmore, 2006). The most accepted hypothesis in the *Candida* field is that fungal eDNA is a by-product derived from cell lysis within the biofilm (personal communications on international conferences). Our preliminary studies on the evaluation of the eDNA electrophoretic mobility in conventional agarose gels containing ethidium bromide showed that eDNA is not similar to genomic DNA, exhibiting a smear profile (ranging approximately from 5000 to 75 bp) (unpublished data). This suggests that *C. albicans* eDNA is derived from cell death (van, V and Pretorius, 2008). However, research is still needed: (i) to establish a possible correlation between cell death (necrosis and/or apoptosis), eDNA release, and biofilm formation, and (ii) to exploit other origin(s) of eDNA.

In addition, the role of eDNA was addressed in terms of biofilm development and antifungal susceptibility. First, *C. albicans* biofilms were formed using a microtiter plate model (Pierce et al., 2008) and the effect of eDNA degradation by DNase and addition of exogenous DNA at different stages of biofilm development was evaluated. These experiments indicated that at later stages of biofilm development: (i) DNase treatment (> 0.03 mg/ml) decreases biofilm biomass (Figure 1A) in comparison with non-treated

biofilms (Figure 1B), and (ii) addition of exogenous DNA (>160 ng/ml) increases biofilm biomass (data not shown). This suggests that eDNA contributes to the maintenance and stability of mature biofilms, but not to their establishment (Martins et al., 2010b).

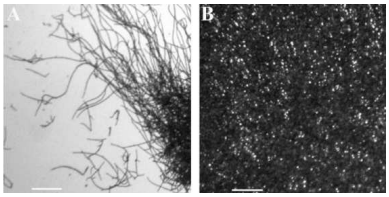


Figure 1: Example of microscopy evaluation of 24-h *C. albicans* biofilms treated with DNase 0.13 mg/ml (A), in comparison with non-treated controls (B).

One of the aspects that may be further exploited relates to the existence and/or role of eDNA derived from biofilm cells but not embedded within the ECM. In fact, it is possible that a portion of the eDNA released by biofilm cells is not trapped by the ECM, being released to the surrounding environment. It may be interesting to address: (i) how circulating eDNA derived from biofilm cells circulates, since it is known that proteins are able to bind DNA (Tamkovich et al., 2008), and (ii) the role of biofilm eDNA in the modulation of the host immune system. A step forward in the research on eDNA in *Candida* species, is the evaluation of the potential use of eDNA as a marker for the diagnostic and/or prognostic of *Candida* infections, similarly to the observed for other *Candida* extracellular components such as β-1,3-glucans (Montagna et al., 2009).

Second, our investigations showed a novel role for eDNA in *C. albicans* biofilms, as a regulator of antifungal susceptibility. Results in Figure 2 show that mature biofilm cells treated with DNase and one polyene drug -amphotericin B- (Figure 2A) or one echinocandin agent -caspofungin- (Figure 2B) and exhibited lower % of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) colorimetric readings in comparison with those treated with antifungal drug only. Contrary to the observed for these antifungals, the susceptibility of *C. albicans* biofilm cells against the azole drug fluconazole was not changed by the addition of DNase (data not shown). These results suggest that DNase increases the effectiveness of these drugs against *C. albicans* biofilms. This is particularly relevant considering biofilm cells increased antifungal resistance in comparison with planktonic counterparts (Chandra et al., 2001). Notably, in the last years the combined use of drugs (Espinel-Ingroff, 2009) or drugs with agents such as enzymes (Kaplan, 2009), has received considerable attention. Advantages of the use of ECM degrading enzymes include their broad spectrum activity, being unlikely the development of antimicrobial resistance, and their use as therapeutic agents (e.g. DNase is already used for cystic fibrosis treatment), suggesting that they may be well tolerated and active when used in a clinical setting. However, it may not be ignored that enzyme-based therapies are expensive and may increase the risk of distal sites colonization due to biofilm dispersion (Kaplan, 2009).

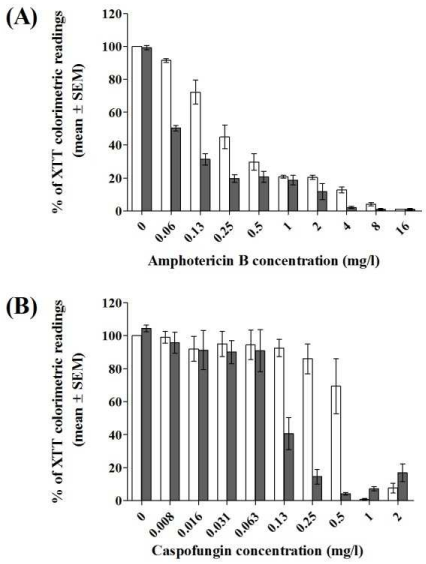


Figure 2: Activity of amphotericin B (A) and caspofungin (B) used alone (white bars) or in combination with DNase 0.13 mg/ml (grey bars) against *C. albicans* 24-h biofilms.

Even though, the potential therapeutic use of ECM degrading enzymes is not a new issue in the *Candida* field, since the enhanced efficacy of the combined use of β-1,3-glucanase and antifungal agents in the eradication of *C. albicans* biofilms has been previously shown by Nett el al. (2007). This corroborates our observations that agents that target processes affecting the biofilm structural integrity may have potential use as therapeutic adjuvants in biofilm treatment, at least as part of antifungal lock-like therapy of devices. Nevertheless, the potential relevance of our findings, requires further in vitro experiments in order: (i) to understand whether the reduced levels of mitochondrial cell activity (% XTT colorimetric readings) represent an effective reduction in metabolism or are resultant of cell dispersion, (ii) to determine the fractional inhibitory concentration index, as a measure of synergy between DNase and these antifungal agents, and (iii) the evaluation of more drugs from each antifungal class. Furthermore, for the consideration of DNase as an anti-candidal enzyme, it is necessary to evaluate eDNA presence and function on an additional number of *C. albicans* strains and other *Candida* species.

Overall, the results of this set of experiments show that eDNA is a component of *C. albicans* biofilm ECM that contributes to biofilm integrity and antifungal resistance. Introducing a new line of research, these studies expanded the current knowledge on *C. albicans* biofilm ECM.

EXTRACELLULAR ALCOHOLS

The second set of molecules selected in our studies comprised extracellular alcohols. These were pointed as targets for our research due to: (i) the recent discovery of the extracellular alcohol farnesol as a quorum sensing molecule in *C. albicans* (Hornby et al., 2001), and (ii) sparse studies suggesting that fungal cells release autoregulatory molecules into the extracellular medium, such as fatty acids (Noverr et al., 2003) or aromatic alcohols (e.g., tyrosol) (Chen et al., 2004). As observed in other *Candida* research areas, the investigation on NCAC species is scarce, and for example not much is known on the role of farnesol in NCAC species. In addition, the identification of extracellular alcohols released by *C. albicans*, and NCAC species, is dependent on appropriate analytical methods. Overcoming this constraint will open the possibility of exploiting the interaction of the identified molecules with virulence traits of *Candida* species.

Previous investigations on the effect of farnesol on NCAC species planktonic cells suggested a species dependent response: (i) in *C. dubliniensis*, dimorphism control, without growth changes (Henriques et al., 2007), and (ii) in *C. parapsilosis*, growth regulation (Rossignol et al., 2007). Thus we aimed at examining the effect of the addition of exogenous farnesol in other NCAC species planktonic cells. Therefore, the effect of farnesol (5 to 150 µM) in *C. glabrata*, *C. krusei*, and *C. tropicalis* planktonic cells was evaluated using a set of methodologies, that included morphology, growth (Henriques et al., 2007) and viability (Coder, 1997) monitoring, and DNA profiling for cell cycle evaluation (Fortuna et al., 2000). Farnesol did not induce morphological changes in the assayed NCAC species (data not shown). As exemplified in Figure 3 for farnesol 100 µM treatment, this alcohol affects *C. glabrata* (Figure 3A), *C. krusei* (Figure 3B), and *C. tropicalis* (Figure 3C) growth. Specifically, farnesol (100 and 150 µM) compromised *C. glabrata* growth, which was not associated with changes in cell survival, although S-G2/M cell cycle phase transition was delayed; (ii) farnesol (50, 100, and 150 µM) delayed *C. krusei* growth without compromising cell viability, but delaying G0/G1 cell cycle phase; (iii) farnesol 100 and 150 µM delayed *C. tropicalis* growth, that was associated with a decrease in cell viability and G0/G1 phase cell cycle phase delay. Notably, very recently, an independent group reported the growth inhibitory effect of farnesol on NCAC species (Weber et al., 2010), although the cytotoxic process was not assessed, contrarily to the evaluation performed in our study. Despite the considerable insights given by our work into the role of farnesol in NCAC species two main questions remain unanswered: first, the existence of farnesol receptors, and second, the potential farnesol targets inside *Candida* cells. As noted earlier, only recently the NCAC species genome sequences were available. From now on, it will be possible to develop for example oligonucleotide microarrays, required for the analysis of global pattern gene expression of NCAC species, for example, under the exposure to farnesol.

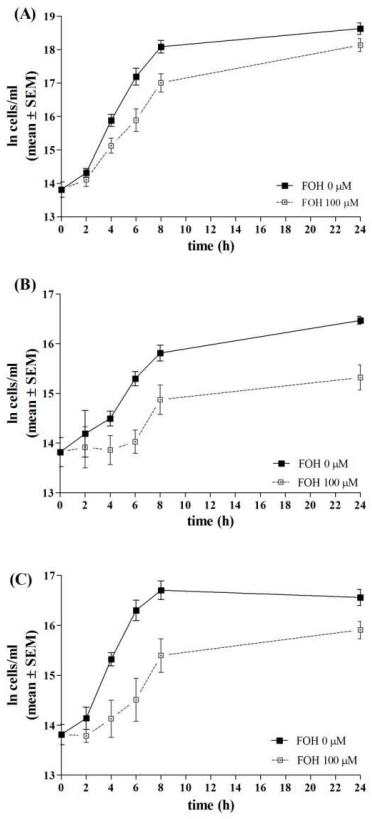


Figure 3: Effect of farnesol on *C. glabrata* (A), *C. krusei* (B), and *C. tropicalis* (C) growth.

Although the regulation of farnesol production by *Candida* species is still under debate, the farnesol levels considered physiologically relevant are below 50 µM (Langford et al., 2009). It should be noted that physiological farnesol levels do not affect *C. glabrata*, *C. krusei*, and *C. tropicalis* growth, but they regulate *C. albicans* and *C. dubliniensis* morphology. This suggests that farnesol acts more likely as a quorum sensing molecule in *C. albicans* and *C. dubliniensis*, regulating a specific virulence trait of these *Candida* species (Sullivan et al., 2005;Calderone and Fonzi, 2001), than in the other *Candida* species.

In addition, ongoing research in our lab suggests that *C. albicans* releases into the extracellular medium other molecules that differentially regulate NCAC species growth. Specifically, *C. albicans* supernatant: (i) does not exhibit major effects on *C. glabrata* and *C. dubliniensis* growth, (ii) promotes *C. krusei* and *C. parapsilosis* cell growth, as indicated by the higher levels of absorbance at 620 nm readings compared with control cells, but (iii) has a growth inhibitory effect on *C. tropicalis* (unpublished data). Moreover, we have reported that *C. albicans* and *C. dubliniensis* supernatants are able to regulate their own morphology (Martins et al., 2007). These findings prompted our research to the characterization of the molecules present in the extracellular medium and the evaluation of their effect.

Using headspace solid-phase microextraction, a microextraction technique based in an equilibrium partition of the compounds between the sample matrix and a polymer-coated fused fiber that can be directly coupled with gas chromatography-mass spectrometer (Arthur and Pawliszyn, 1990), it was established, for the first time, the profile of multiple extracellular alcohols in *Candida* species (Martins et al., 2007;Martins et al., 2010a). Therefore, in an unified and detailed study along time (from 24 to 96 h), a group of extracellular alcohols- isoamyl alcohol, 2-phenylethanol (phenylethanol), 1-dodecanol (dodecanol), *E*-nerolidol (nerolidol), and farnesol were identified and quantified in planktonic and biofilm supernatants of *C. albicans* and *C. dubliniensis* (Table 2). In these samples, dodecanol was found in trace quantities (Martins et al., 2007). Although along time the alcohols secretion profiles were species and culture mode (planktonic vs biofilm) specific (Table 2), it was not possible to establish a biofilm fingerprint concerning these alcohols identity. Thus, as an initial approach to get insights into extracellular alcohols produced by other *Candida* species, only planktonic supernatants were analyzed using the same methodology. These studies showed that *C. parapsilosis* and *C. tropicalis* 24-h supernatants contained farnesol, isoamyl alcohol, phenylethanol, and dodecanol, but not nerolidol (Martins et al., 2010a).

It has been suggested that the majority of the molecules secreted by microorganisms are able to modulate physiological functions, although the observed effects are not always derived from a quorum sensing circuit but many times resultant from processes of compound metabolisation or detoxification (Winzer et al., 2002). It is interesting to note that the identified extracellular alcohols have a volatile nature, which may help the signal diffusion, communication between cells, and regulation of virulence factors. To get insights into the interaction of the identified extracellular alcohols with virulence traits of *Candida* species, the effect of the addition of commercial formulations of the identified alcohols, at physiological and/or supraphysiological concentrations [using as reference the *C. albicans* and *C. dubliniensis* produced levels (Martins et al., 2007)], was examined in terms of *Candida* species morphogenesis, growth regulation (Martins et al., 2007), biofilm formation (Martins et al., 2010a), and in the progression of disseminated candidiasis.

First, from our studies on the effect of *C. albicans* and *C. dubliniensis* extracellular alcohols in their planktonic cells morphology (Martins et al., 2007), it was found that under filamentation inducing conditions, these compounds inhibit the yeast to filamentous form conversion (Table 3), calculated as the number of hyphae or pseudohyphae in the presence (HPpresA) and absence of each alcohol (HPabsA) (% inhibition= [(HPabsA – HppresA)/ HPabsA]*100). For the indicated alcohol levels *C. albicans* and *C. dubliniensis* growth was not affected (data not shown). These findings added isoamyl alcohol, phenylethanol, dodecanol, and nerolidol as *Candida* species secreted substances involved in the morphogenesis regulation. Although the intracellular targets of farnesol in *C. albicans* began to be dissected [reviewed by (Langford et al., 2009)] the intracellular targets of isoamyl alcohol, phenylethanol, and nerolidol are not known. To elucidate this issue, and in a first approach, the evaluation of the expression of genes involved in key pathways of morphogenesis (Biswas et al., 2007) may be performed. Furthermore, the knowledge on *C. dubliniensis* filamentation regulation by extracellular alcohols is even scarcer than in *C. albicans*. Thus, it

Table 2: Concentration of extracellular alcohols in *C. albicans* and *C. dubliniensis* planktonic and biofilm supernatants

	Alcohol	Planktonic culture				Biofilm culture			
		Culture time (h)				Culture time (h)			
		24	48	72	96	24	48	72	96
C. albicans	Isoamyl alcohol (µmol/g cell dry weight)	64.09	58.23	59.36	57.91	12.94	45.17	72.49	46.12
	Phenylethanol (µmol/g cell dry weight)	7.37	18.44	24.62	43.22	5.29	29.39	37.12	88.77
	Nerolidol (nmol/g cell dry weight)	0.70	2.42	2.03	1.96	0.46	2.94	7.53	8.80
	Farnesol (nmol/g cell dry weight)	0.02	0.59	0.25	11.08	0.02	0.16	0.50	5.08
C. dubliniensis	Isoamyl alcohol (µmol/g cell dry weight)	68.79	42.15	20.00	32.90	15.32	25.90	22.70	37.99
	Phenylethanol (µmol/g cell dry weight)	15.40	14.35	19.57	25.80	7.26	41.87	31.46	67.13
	Nerolidol (nmol/g cell dry weight)	0.74	2.29	0.79	0.64	1.30	3.07	4.09	3.80
	Farnesol (nmol/g cell dry weight)	0.07	2.13	0.20	0.84	0.15	1.59	0.65	1.05

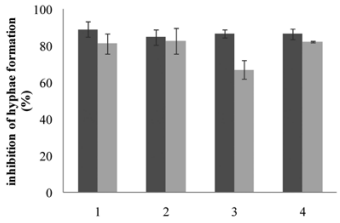
may be particularly interesting to elucidate these pathways, since it has been suggested that the dimorphism regulation may differ between *C. albicans* and *C. dubliniensis* (Sullivan et al., 2005).

Table 3: Effect of secreted alcohols on the filamentation inhibition of planktonic *C. albicans* and *C. dubliniensis*

Alcohol	Concentration	*C. albicans* % Hyphae inhibition	*C. dubliniensis* % Pseudohyphae inhibition
Isoamyl alcohol	23 mM	95.8 ± 3.0	92.4 ± 2.1
	46 µM	68.4 ± 12.3	70.7 ± 15.6
Phenylethanol	500 µM	95.3 ± 1.23	91.5 ± 10.7
	5 µM	71.4 ± 15.7	72.9 ± 17.0
Dodecanol	2 µM	69.9 ± 16.8	74.5 ± 9.6
	2 nM	57.8 ± 14.3	54.1 ± 12.1
Nerolidol	1.5 µM	64.8 ± 4.7	64.6 ± 7.8
	1.5 nM	51.1 ± 9.3	68.3 ± 5.0
Farnesol	1.5 µM	67.5 ± 10.1	74.1 ± 17.8
	1.5 nM	63.5 ± 10.8	57.8 ± 4.8

Second, we screened these compounds for their ability to regulate *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* sessile cells evaluating the effect of the individual addition of the alcohols after 3 h of adhesion and 48 h of biofilm formation. After 24 h, biofilms were analyzed in terms of cellular mitochondrial activity (XTT assay) and total biomass (crystal violet assay). It was found that (i) isoamyl alcohol elicited antibiofilm activity against *C. albicans* and *C. dubliniensis*, and a heterogeneous activity against *C. tropicalis* biofilm cells, (ii) phenylethanol showed antibiofilm activity against *C. tropicalis*, (iii) dodecanol elicited a probiofilm activity in *C. parapsilosis* but antibiofilm activity in *C. tropicalis*, (iv) nerolidol showed an antibiofilm activity against *C. parapsilosis* and *C. tropicalis*, and (v) farnesol reduced *C. albicans* and *C. dubliniensis* biofilm (Martins et al., 2010a). These results show for the first time, that other alcohol compounds, besides farnesol, regulate *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* biofilm development. Concerning the effect of the evaluated alcohols on biofilm development, further studies are still required to evaluate: (i) the production of the different extracellular alcohols by *C. parapsilosis* and *C. tropicalis* biofilm cells, (ii) the impact of increasing the range of concentrations tested when these alcohols showed an effect, and (iii) other parameters such as biofilm cells morphology, and ECM composition, including eDNA. In addition, it would be interesting to elucidate how the cells in different regions of the biofilm respond to the addition of the alcohols. This can now be accomplished by cryosectioning the biofilms and using capture microdissection microscopy coupled with multiplex quantitative real-time reverse transcriptase polymerase chain reaction (Lenz et al., 2008).

Third, as these alcohols were not individually identified in the supernatant, they may not act isolated but rather as part of a network. To further elucidate the biological relevance of these alcohols, *C. albicans* and *C. dubliniensis* 96-h planktonic and biofilm supernatants were mimicked using synthetic mixtures (cocktail solutions) containing commercial formulations of isoamyl alcohol, phenylethanol, nerolidol, and farnesol at the concentrations determined (Martins et al., 2007). In general, the in vitro effect of the cocktail mixtures, on *C. albicans* (Figure 4) and *C. dubliniensis* (data not shown) filamentation inhibition was similar to that of the corresponding supernatants. The exception was the cocktail solution mimicking *C. dubliniensis* planktonic supernatant (Figure 4, condition 3), suggesting that other morpho-regulatory molecules may be present in these fractions.



1- *C. albicans* planktonic supernatant
2- *C. albicans* biofilm supernatant
3- *C. dubliniensis* planktonic supernatant
4- *C. dubliniensis* biofilm supernatant

Figure 4: Effect of 96-h supernatants (dark grey bars) and corresponding cocktail solutions (light grey bars) on the morphology of 12-h planktonic cells of *C. albicans*.

In addition, an indication of the in vivo relevance of these alcohols was shown during infection in a well established murine model of hematogenously disseminated candidiasis (Clancy et al., 2009). At the time of infection, mice were injected intraperitoneally with the Cocktail solution simulating the composition of alcohols present in a *C. albicans* culture supernatant (1 ml; 94 µM isoamyl alcohol, 70 µM phenylethanol, 3.2 nM nerolidol and 18 nM farnesol). This study showed an increased survival (Figure 5) and decreased kidney fungal burdens (data not shown) for that Cocktail treated mice vs control ones (injected with Vehicle only). In addition, histological observations suggested that the Cocktail, to some extent, has an inhibitory effect on cell proliferation and filamentation within the tissues (data not shown). It is still to explore how the Cocktail solution interferes with the host immune response, thus modulating the disease progression. This could be achieved by profiling cytokines with systems such as the Bio-Plex®, that allow the simultaneously quantification of several mouse cytokines. In addition, it would be interesting to elucidate the contribution of each individual alcohol in the progression of the disseminated disease. Moreover, evidences in literature suggest that the role of farnesol in vivo depends on the disease model (Navarathna et

al., 2007;Hisajima et al., 2008). In light of our findings it would be of interest: (i) to test the effect of the Cocktail solution in the recently developed animal biofilm models (Ricicova et al., 2009;Lazzell et al., 2009), and (ii) to ascertain the in vivo distribution and metabolism of these compounds, which can be achieved by the use of emerging imaging techniques such as mass spectrometry imaging (Sugiura and Setou, 2010).

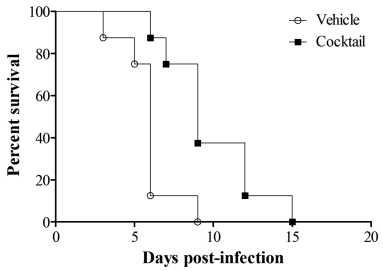


Figure 5: Effect of the administration of *Candida albicans* autoregulatory alcohols on mice survival.

Although these studies began to dissect the regulation of *Candida* species biology through autoregulatory molecules, there are additional points that could be addressed throughout, such as: (i) the elucidation of the metabolic pathways underlying the production of these compounds, for example by the use of ^{13}C nuclear magnetic resonance spectroscopy, as previously performed for *S. cerevisiae* (Dickinson, 2008), (ii) the study of the physiological conditions that regulate the production of the these alcohols in the planktonic and biofilm lifestyle, (iii) how the externally added compounds are metabolized by the cells, (iv) the evaluation of the effect of the alcohols on other virulence attributes, as adhesins, proteolytic and hydrolytic enzymes secretion (Trofa et al., 2008;Calderone and Fonzi, 2001;Fidel, Jr. et al., 1999;Sullivan et al., 2005), and (v) the evaluation of an higher number of strains of each *Candida* species.

Overall, the results of this set of experiments report that other alcohol molecules, besides farnesol are produced by *Candida* species, and that these molecules are able to modulate filamentation, biofilm development, and disease pathogenesis, depending on the alcohol and on the *Candida* species. In short term, these findings increased our current understanding on the regulation of physiology and virulence traits of *Candida* species through the secretion of molecules. However, it cannot be disregarded that in a natural environment, the signal molecules may be washed out and the concentration of the compounds is a balance between their production, degradation or half-life time (Horswill et al., 2007) and site of colonization/ infection. So, one of the major challenges in the future is to be able to distinguish the differential production of molecules by the different species in different environments, in order to establish the comprehensive and accurate dynamic changes of molecules within a cell, which can only be achieved by the use of modeling tools (Zhang et al., 2010).

CONCLUSIONS

In summary, the information resulting from this thesis contributes to a better understanding of the extracellular milieu, not only of *C. albicans* but also of NCAC species. In short term, we increased our knowledge on specific components of *Candida* species extracellular medium- eDNA and alcohol compounds- and on their interaction with *Candida* species phenotype. In long term, the insights achieved can open the doors to other investigations that can lead to the development of treatment and/ or diagnostic strategies to combat disseminated candidiasis.

REFERENCES

Al-Fattani MA and Douglas LJ. 2006. "Biofilm Matrix of *Candida albicans* and *Candida tropicalis*: Chemical Composition and Role in Drug Resistance." *Journal of Medical Microbiology* 55, 999-1008.

Arthur C and Pawliszyn J. 1990. "Solid Phase Microextraction with Thermal Desorption using Fused Silica Optical Fibers." *Analytical Chemistry* 62, 2145-2148.

Biswas S, Van DP, and Datta A. 2007. "Environmental Sensing and Signal Transduction Pathways Regulating Morphopathogenic Determinants of *Candida albicans*." *Microbiology and Molecular Biology Reviews* 71, 348-376.

Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJ, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PW, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA, Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KA, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MP, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NA, Lorenz MC, Birren BW, Kellis M, and Cuomo CA. 2009. "Evolution of Pathogenicity and Sexual Reproduction in Eight *Candida* genomes." *Nature* 459, 657-662.

Calderone RA and Fonzi WA. 2001. "Virulence Factors of *Candida albicans*." *Trends in Microbiology* 9, 327-335.

Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, and Ghannoum MA. 2001. "Biofilm Formation by the Fungal Pathogen *Candida albicans*: Development, Architecture, and Drug Resistance." *Journal of Bacteriology* 183, 5385-5394.

Chen H, Fujita M, Feng Q, Clardy J, and Fink GR. 2004. "Tyrosol is a Quorum-Sensing Molecule in *Candida albicans*." *Proceedings of the National Academy of Sciences of the United States of America* 101, 5048-5052.

Clancy CJ, Cheng S, and Nguyen M. 2009. "Animal Models of Candidiasis." *Methods in Molecular Biology* 499, 65-76.

Coder D. 1997. "Assessment of Cell Viability." *Current Protocols in Cytometry* 9.2.1-9.2.14.

Coenye T. 2010. "Response of Sessile Cells to Stress: from Changes in Gene Expression to Phenotypic Adaptation." *FEMS Immunology and Medical Microbiology* 59, 239-252.

Dickinson JR. 2008. "Filament Formation in *Saccharomyces cerevisiae*-a Review." *Folia Microbiologica* 53, 3-14.

Dimitrova P, Yordanov M, Danova S and Ivanovska N. 2008. "Enhanced Resistance Against Systemic *Candida albicans* Infection in Mice Treated with *C. albicans* DNA." *FEMS Immunology and Medical Microbiology* 53, 231-236.

Draghi JA and Turner PE. 2006. "DNA Secretion and Gene-Level Selection in Bacteria." *Microbiology* 152, 2683-2688.

Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De MJ, Marck C, Neuveglise C, Talla E, Goffard N, Frangeul L, Aigle M, Anthouard V, Babour A, Barbe V, Barnay S, Blanchin S, Beckerich JM, Beyne E, Bleykasten C, Boisrame A, Boyer J, Cattolico L, Confanioleri F, De DA, Despons L, Fabre E, Fairhead C, Ferry-Dumazet H, Groppi A, Hantraye F, Hennequin C, Jauniaux N, Joyet P, Kachouri R, Kerrest A, Koszul R, Lemaire M, Lesur I, Ma L, Muller H, Nicaud JM, Nikolski M, Oztas S, Ozier-Kalogeropoulos O, Pellenz S, Potier S, Richard GF, Straub ML, Suleau A, Swennen D, Tekaia F, Wesolowski-Louvel M, Westhof E, Wirth B, Zeniou-Meyer M, Zivanovic I, Bolotin-Fukuhara M, Thierry A, Bouchier C, Caudron B, Scarpelli C, Gaillardin C, Weissenbach J, Wincker P, and Souciet JL. 2004. "Genome Evolution in Yeasts." *Nature* 430, 35-44.

Espinel-Ingroff A. 2009. "Novel Antifungal Agents, Targets or Therapeutic Strategies for the Treatment of Invasive Fungal Diseases: a Review of the Literature (2005-2009)." *Revista Iberoamericana de Micología* 26,15-22.

Fidel PL, Jr., Vazquez JÁ, and Sobel JD. 1999. "*Candida glabrata*: Review of Epidemiology, Pathogenesis, and Clinical Disease with Comparison to *C. albicans*." *Clinical Microbiology Reviews* 12, 80-96.

Fortuna M, Sousa MJ, Corte-Real M, Leão C, Salvador A, and Sansonetty F. 2000. "Cell Cycle Analysis of Yeasts." *Current Protocols in Cytometry* 11.13.1-11.13.9.

Frisvad JC, Larsen TO, de VR, Meijer M, Houbraken J, Cabanes FJ, Ehrlich K, and Samson RA. 2007. "Secondary Metabolite Profiling, Growth Profiles and Other Tools for Species Recognition and Important *Aspergillus* Mycotoxins." *Studies in Mycology* 59, 31-37.

Haynes K. 2001. "Virulence in *Candida* species." *Trends in Microbiology* 9, 591-596.

Hazen KC. 1995. "New and Emerging Yeast Pathogens." *Clinical Microbiology Reviews* 8, 462-478.

Hennen CR. 2009. "Pharmacoeconomic Evaluations of Antifungal Therapies." *Current Medical Research and Opinion* 25, 1751-1758.

Henriques M, Martins M, Azeredo J, and Oliveira R. 2007. "Effect of Farnesol on *Candida dubliniensis* Morphogenesis." *Letters in Applied Microbiology* 44, 199-205.

Hisajima T, Maruyama N, Tanabe Y, Ishibashi H, Yamada T, Makimura K, Nishiyama Y, Funakoshi K, Oshima H, and Abe S. 2008. "Protective Effects of Farnesol Against Oral Candidiasis in Mice." Microbiology and Immunology 52, 327-333.

Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P, and Nickerson KW. 2001. "Quorum Sensing in the Dimorphic Fungus *Candida albicans* is Mediated by Farnesol." *Applied and Environmental Microbiology* 67, 2982-2992.

Horswill AR, Stoodley P, Stewart PS, and Parsek MR. 2007. "The Effect of the Chemical, Biological, and Physical Environment on Quorum Sensing in Structured Microbial Communities." *Analytical and Bioanalytical Chemistry* 387, 371-380.

Jackson AP, Gamble JA, Yeomans T, Moran GP, Saunders D, Harris D, Aslett M, Barrell JF, Butler G, Citiulo F, Coleman DC, de Groot PW, Goodwin TJ, Quail MA, McQuillan J, Munro CA, Pain A, Poulter RT, Rajandream MA, Renauld H, Spiering MJ, Tivey A, Gow NA, Barrell B, Sullivan DJ, and Berriman M. 2009. "Comparative Genomics of the Fungal Pathogens *Candida dubliniensis* and *C. albicans*." *Genome Research* 19, 2231-2244.

Jewett MC, Hofmann G, and Nielsen J. 2006. "Fungal Metabolite Analysis in Genomics and Phenomics." *Current Opinion in Biotechnology* 17, 191-197.

Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW, and Scherer S. 2004. "The Diploid Genome Sequence of *Candida albicans*." *Proceedings of the National Academy of Sciences of the United States of America* 101, 7329-7334.

Kaplan JB. 2009. "Therapeutic Potential of Biofilm-Dispersing Enzymes." *The International Journal of Artificial Organs* 32, 545-554.

Kasai M, Francesconi A, Petraitiene R, Petraitis V, Kelaher AM, Kim HS, Meletiadis J, Sein T, Bacher J, and Walsh TJ. 2006. "Use of Quantitative Real-Time PCR to Study the Kinetics of Extracellular DNA Released from *Candida albicans*, with Implications for Diagnosis of Invasive Candidiasis." *Journal of Clinical Microbiology* 44, 143-150.

Langford ML, Atkin AL, and Nickerson KW. 2009. "Cellular Interactions of Farnesol, a Quorum-Sensing Molecule Produced by *Candida albicans*." *Future Microbiology* 4, 1353-1362.

Lazzell AL, Chaturvedi AK, Pierce CG, Prasad D, Uppuluri P, and Lopez-Ribot JL. 2009. "Treatment and Prevention of *Candida albicans* Biofilms with Caspofungin in a Novel Central Venous Catheter Murine Model of Candidiasis." *Journal of Antimicrobial Chemotherapy* 64, 567-570.

Lenz AP, Williamson KS, Pitts B, Stewart PS, and Franklin MJ. 2008. "Localized Gene Expression in *Pseudomonas aeruginosa* Biofilms." *Applied and Environmental Microbiology* 74, 4463-4471.

Lewis RE. 2009. "Overview of the Changing Epidemiology of Candidemia." *Current Medical Research and Opinion* 25, 1732-1740.

Li L, Redding S and Dongari-Bagtzoglou A. 2007. "*Candida glabrata*: an Emerging Oral Opportunistic Pathogen." *Journal of Dental Research* 86, 204-215.

Loreto ES, Scheid LA, Nogueira CW, Zeni G, Santurio JM, and Alves SH. 2010. "*Candida dubliniensis*: Epidemiology and Phenotypic Methods for Identification." *Mycopathologia* 169, 431-443.

Martins M, Henriques M, Azeredo J, Rocha SM, Coimbra MA, and Oliveira R. 2007. "Morphogenesis Control in *Candida albicans* and *Candida dubliniensis* Through Signaling Molecules Produced by Planktonic and Biofilm Cells." *Eukaryotic Cell* 6, 2429-2436.

Martins M, Henriques M, Azeredo J, Rocha SM, Coimbra MA, and Oliveira R. 2010a. "*Candida* Species Extracellular Alcohols: Production and Effect in Sessile Cells." *Journal of Basic Microbiology* in press.

Martins M, Uppuluri P, Thomas DP, Cleary IA, Henriques M, Lopez-Ribot JL, and Oliveira R. 2010b. "Presence of Extracellular DNA in the *Candida albicans* Biofilm Matrix and its Contribution to Biofilms." *Mycopathologia* 169, 23-331.

Miyazato A, Nakamura K, Yamamoto N, Mora-Montes HM, Tanaka M, Abe Y, Tanno D, Inden K, Gang X, Ishii K, Takeda K, Akira S, Saijo S, Iwakura Y, Adachi Y, Ohno N, Mitsutake K, Gow NA, Kaku M, and Kawakami K. 2009. "Toll-like receptor 9-Dependent Activation of Myeloid Dendritic Cells by Deoxynucleic Acids from *Candida albicans*." *Infection and Immunity* 77, 3056-3064.

Montagna MT, Caggiano G, Borghi E, and Morace G. 2009. "The role of the Laboratory in the Diagnosis of Invasive Candidiasis." *Drugs* 69, 59-63.

Nantel A, Dignard D, Bachewich C, Harcus D, Marcil A, Bouin AP, Sensen CW, Hogues H, van het HM, Gordon P, Rigby T, Benoit F, Tessier DC, Thomas DY, and Whiteway M. 2002. "Transcription Profiling of *Candida albicans* Cells Undergoing the Yeast-to-Hyphal Transition." *Molecular Biology of the Cell* 13, 3452-3465.

Navarathna DH, Hornby JM, Krishnan N, Parkhurst A, Duhamel GE, and Nickerson KW. 2007. "Effect of Farnesol on a Mouse Model of Systemic Candidiasis: a DPP3 Knock Out Mutant of *Candida albicans*." *Infection and Immunity* 75, 1609-1618.

Nett J, Lincoln L, Marchillo K, Massey R, Holoyda K, Hoff B, VanHandel M, and Andes D. 2007. "Putative Role of β-1,3glucans in *Candida albicans* Biofilm Resistance." *Antimicrobial Agents and Chemotherapy* 51, 510-520.

Noverr MC, Erb-Downward JR, and Huffnagle GB. 2003. "Production of Eicosanoids and Other Oxylipins by Pathogenic Eukaryotic Microbes." *Clinical Microbiology Reviews* 16, 517-533.

Oldiges M, Lutz S, Pflug S, Schroer K, Stein N, and Wiendahl C. 2007. "Metabolomics: Current State and Evolving Methodologies and Tools." *Applied Microbiology and Biotechnology* 76, 495-511.

Pfaller MA and Diekema DJ. 2007. "Epidemiology of Invasive Candidiasis: a Persistent Public Health Problem." *Clinical Microbiology Reviews* 20, 133-163.

Pierce CG, Uppuluri P, Tristan AR, Wormley FL, Jr., Mowat E, Ramage G, and Lopez-Ribot JL. 2008. "A Simple and Reproducible 96-well Plate-Based Method for the Formation of Fungal Biofilms and its

Application to Antifungal Susceptibility Testing." *Nature Protocols* 3, 1494-1500.

Ricicova M, Kucharikova S, Tournu H, Hendrix J, Bujdakova H, Van EJ, Lagrou K, and Van DP. 2009. "*Candida albicans* Biofilm Formaton in a New in vivo Rat Model." *Microbiology* 156, 909-919.

Rossignol T, Logue ME, Reynolds K, Grenon M, Lowndes NF, and Butler G. 2007. "Transcriptional Response of *Candida parapsilosis* Following Exposure to Farnesol." *Antimicrobial Agents and Chemotherapy* 51, 2304-2312.

Samaranayake YH and Samaranayake LP. 1994. "*Candida krusei*: Biology, Epidemiology, Pathogenicity and Clinical Manifestations of an Emerging Pathogen." *Journal of Medical Microbiology* 41, 295-310.

Sauer U, Heinemann M, and Zamboni N. 2007. "Genetics. Getting Closer to the Whole Picture." *Science 316*, 550-551.

Skrzypek MS, Arnaud MB, Costanzo MC, Inglis DO, Shah P, Binkley G, Miyasato SR, and Sherlock G. 2010. "New Tools at the Candida Genome Database: Biochemical Pathways and Full-Text Literature Search." *Nucleic Acids Research* 38, D428-D432.

Spoering AL and Gilmore MS. 2006. "Quorum Sensing and DNA Release in Bacterial Biofilms." *Current Opinion in Microbiology* 9, 133-137.

Sugiura Y and Setou M. 2010. "Imaging Mass Spectrometry for Visualization of Drug and Endogenous Metabolite Distribution: Toward in situ Pharmacometabolomes." *Journal of Neuroimmune Pharmacology* 5, 31-43.

Sullivan DJ, Moran GP, and Coleman DC. 2005. "*Candida dubliniensis*: Ten years on." *FEMS Microbiology Letters* 253, 9-17.

Tamkovich SN, Vlassov VV, and Laktionov PP. 2008. "Circulating Deoxyribonucleic Acids in Blood and their Using in Medical Diagnostics." *Molecular Biology* 42, 12-23.

Thomas DP, Pitarch A, Monteoliva L, Gil C, and Lopez-Ribot JL. 2006. "Proteomics to Study *Candida albicans* Biology and Pathogenicity." *Infectious Disorders Drug Targets* 6, 335-341.

Trofa D, Gacser A and Nosanchuk JD. 2008. "*Candida parapsilosis*, an Emerging Fungal Pathogen." *Clinical Microbiology Reviews* 21, 606-625.

van d, V and Pretorius PJ. 2008. "Circulating DNA. Its Origin and Fluctuation." *Annals of the New York Academy of Sciences* 1137, 18-26.

Villas-Boas SG, Rasmussen S, and Lane GA. 2005. "Metabolomics or Metabolite profiles?" *Trends in Biotechnology* 23, 385-386.

Weber K, Schulz B, and Ruhnke M. 2010. "The Quorum-Sensing Molecule *E,E*-farnesol-its Variable Secretion and its Impact on the Growth and Metabolism of *Candida* Species." *Yeast* 27, 727-739.

Winzer K, Hardie KR, and Williams P. 2002. "Bacterial Cell-to-Cell Communication: Sorry, Can't Talk Now-Gone to Lunch!" *Current opinion in Microbiology* 5, 216-222.

Yordanov M, Dimitrova P, Danova S and Ivanovska N. 2005. "*Candida albicans* Double-Stranded DNA Can Participate in the Host Defense Against Disseminated Candidiasis." *Microbes and Infection* 7, 178-186.

Zhang W, Li F, and Nie L. 2010. "Integrating Multiple 'omics' Analysis for Microbial Biology: Application and Methodologies." *Microbiology* 156, 287-301.

AUTHORS BIOGRAPHIES

ROSÁRIO OLIVEIRA ROSÁRIO OLIVEIRA graduated in Chemical Engineering at University of Luanda, in 1975. She obtained her PhD degree in Engineering Science in 1991 at the University of Minho. She is currently a full professor at the Department of Biological Engineering of the University of Minho. She is the head of the master degree in Environmental Management and head of the Biofilm research group. Her main research interests are in microbiology and environmental sciences, and animal cell culture technology. Her e-mail is roliveira@deb.uminho.pt and her web page is http://www.deb.uminho.pt/pessoas/en/docente.aspx?id=36

MARIANA HENRIQUES is an invited assistant at the Department of Biological Engineering at University of Minho. Her main research interests are *Candida* species virulence factors, monoclonal antibodies production processes, and characterization of biomaterials. In these areas, she has published 34 peer-reviewed articles and several book chapters. Her email address is mcrh@deb.uminho.pt, and her web page can be found at http://www.deb.uminho.pt/pessoas/docente.aspx?id=28

MARGARIDA MARTINS graduated in Biochemistry from the University of Porto in 2002. She has started her scientific career in Microbiology developing molecular tools to study *Paracoccidioides brasiliensis* for her master thesis. In 2005, Margarida moved to the *Candida* field and started her PhD one year later. She has published ten peer-reviewed articles and one book chapter. As an international exchange visitor student she received training in Paul Hooykaas's Lab (Netherlands), José Lopez-Ribot's Lab (USA), and Neil Gow's Lab (UK). Her email address is margarida.martins@deb.uminho.pt, and her web page can be found at http://www.deb.uminho.pt/pessoas/investigadores/pid.aspx?id=67