This work was supported by the Portuguese Foundation for Science and Technology (FCT) through the PhD grant SFRH/BD/111645/2015 and the strategic funding of UID/BIO/04469/2019 unit and BioTecNorte operation [NORTE-01-0145-FEDER-000004] funded by the European Regional Development Fund, under the scope of Norte2020 - Programa Operacional Regional do Norte.

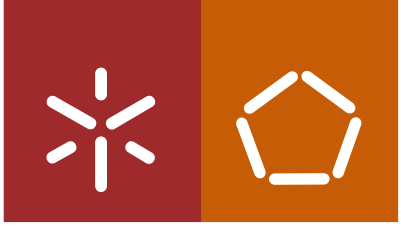
Candida species vaginal virulence determinants: role of environmental factors
Bruna Fernandes Gonçalves
UMinho | 2019

Universidade do Minho
Escola de Engenharia

Bruna Fernandes Gonçalves

Candida species vaginal virulence determinants: role of environmental factors

July 2019



Universidade do Minho
Escola de Engenharia

Bruna Fernandes Gonçalves

Candida species vaginal virulence determinants: role of environmental factors

Dissertation for PhD degree in Chemical and Biological Engineering

Work supervised by:
Dr. Mariana Henriques
Dr. Sónia Silva

July 2019

Agradecimentos

Por trás do sucesso na realização deste trabalho, estão determinadas pessoas cuja contribuição foi fundamental. Gostaria de expressar o meu profundo reconhecimento aos que me acompanharam nesta etapa, pela amizade, apoio, elucidação, disponibilidade, prontidão e transmissão de conhecimentos que foram fulcrais para mim. A todos, muito obrigada.

À professora Mariana, quero agradecer a excelente orientação, disponibilidade e transmissão de conhecimento que foram essenciais para o sucesso deste trabalho. Os seus elogios e incentivos constantes deram-me força para continuar, apesar das muitas dificuldades. Mas há muito mais a agradecer à professora Mariana...a AMIZADE, compreensão, auxílio e constante preocupação nos momentos difíceis, é pouco do MUITO que tenho a agradecer. Quando o mundo desabou não uma, nem duas, mas muitas vezes, quando eu achava que estava sozinha...a professora Mariana estava lá!!!

À Sónia agradeço a amizade, total disponibilidade e orientação excelente. Obrigada por todo o apoio e preocupação constantes. É incrível como estás sempre totalmente disponível para ouvir, para ajudar...adoro a tua alegria, é contagiante!! Muito me aturaste... obrigada por me ajudares a perceber que há coisas que podemos relativizar.

À Priscila, com quem tive o prazer de trabalhar e sobretudo aprender, agradeço toda a ajuda prestada e conhecimento transmitido. Além disso, muito obrigada pela simpatia, amizade e disponibilidade para me ouvires.

À professora Joana agradeço o conhecimento transmitido, preocupação e simpatia constantes.

A todos os membros do grupo das "Candidas", Daniela, Nuno, Liliana, Fernanda, Elisa, Andreia e Isabel, muito obrigada pela alegria, simpatia e auxílio laboratorial sempre que necessário.

A todos do grupo dos "Biofilmes", em especial à Ana, Ivone, Vânia, Graça, Rodrigo, Luís, Sílvio, Pilar, Daniela, Mariana, Diana, Diana Vilas-Boas e Joana, obrigada pela simpatia.

Ao Nuno Mira, agradeço a orientação no estudo dos arrays que melhorou significativamente o trabalho e o meu conhecimento sobre análise de resultados desta técnica.

À minha irmãzinha agradeço a alegria e à kuska o amor incondicional e felicidade.

Mãe, obrigada por sempre teres incentivado e elogiado o meu percurso académico e sei que, onde quer que estejas, mais do que ninguém, estás muito orgulhosa de mim.

Ao Ricardo, agradeço o carinho, apoio constante e compreensão por todo o tempo que não lhe dediquei como tanto merecia e acima de tudo obrigada pelo amor, essencial para o sucesso de qualquer trabalho científico..."Una mitad tan triste, la otra mitad riendo, ahora lo entendo estoy viviendo" (Nach).

A morte é a curva da estrada,
Morrer é só não ser visto.
Se escuto, eu te oiço a passada
Existir como eu existo.

A terra é feita de céu.
A mentira não tem ninho.
Nunca ninguém se perdeu.
Tudo é verdade e caminho.

Fernando Pessoa (poesia ortónima)

Dedicado aos meus queridos pais*, in memoriam*.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Candida species vaginal virulence determinants: role of environmental factors

Abstract

Candida species are relevant opportunistic fungi in the vaginal tract, where they may transform a symptomless colonization into an infection, vulvovaginal candidiasis (VVC). The high incidence of VVC, its negative consequences and the increase of antifungal failure in its treatment, make it crucial to increase the knowledge on vaginal virulence of the most common *Candida* species in VVC, *Candida albicans* and *Candida glabrata*. Thus, this investigation aimed to analyse the modulation of relevant *Candida* virulence factors by specific vaginal conditions, as the acidic environment and presence of hormones, as well as the identification of potential vaginal virulence determinants.

The first step of this work was the analysis of pH influence on the biofilm development of *C. albicans* and *C. glabrata* vaginal isolates. The pH was found to affect biofilms' biomass, structure and matrix composition of both species, being *C. glabrata* biofilm formation favored at acidic conditions and in *C. albicans* at neutral pH. *Candida glabrata* acidic biofilms were further analysed, focusing on matrix production, which was verified to be negatively regulated by the Zap1 transcription factor. Zap1 blocked the secretion of 31 proteins, some with a potential role on carbohydrate matrix delivery. Zap1 was also found to induce the secretion of 134 proteins, revealing a potential role on the modulation of *C. glabrata* matrix proteomic profile by acidic conditions, reporting 397 proteins, for the first time, in the matrix.

The influence of reproductive hormones, progesterone and β-estradiol, on *C. albicans* and *C. glabrata* vaginal isolates at acidic conditions was also evaluated. The hormones led to a dose-dependent increase of cells' resistance to hydrogen peroxide and azoles, in both species, and were also found to affect *C. albicans* biofilm formation and filamentation but not *C. glabrata* biofilm development. Consistently, a transcriptomic analysis of *C. albicans* biofilms developed in the presence of progesterone led to the identification of 166 repressed genes including, among others, the regulator Tec1 and many of its target genes, and also 54 up-regulated genes, including the efflux pumps Cdr1 and Cdr2. The transcriptomic profile of *C. albicans* acidic biofilms was also analysed in the absence of hormone, being revealed 286 genes not previously associated with biofilm formation in *C. albicans*, many regulated by Sfl1, which was found to be essential for biofilm formation at acidic conditions.

Overall this study shows that vaginal conditions have a species-specific modulation of *Candida* virulence factors, being revealed new putative vaginal virulence determinants, thus contributing to a better knowledge of *Candida* vaginal pathogenicity.

Key words: *Candida* species, vaginal environment, virulence determinants, vulvovaginal candidiasis.

Determinantes de virulência vaginal de espécies de *Candida*: papel de fatores ambientais

Sumário

As espécies de *Candida* são fungos oportunistas no trato vaginal, onde podem transformar uma colonização assintomática numa infecção, a candidíase vulvovaginal (CVV). A elevada incidência da CVV e a dificuldade no seu tratamento, tornam crucial o aumento do conhecimento sobre a virulência das espécies de *Candida* mais comuns na VVC, *Candida albicans* e *Candida glabrata*. Assim, este trabalho teve como principal objetivo analisar a modulação dos fatores virulência de *Candida* mais relevantes pelas condições do ambiente vaginal, incluindo o ambiente ácido e a presença de hormonas, e também identificar potenciais determinantes de virulência vaginal.

O primeiro passo deste trabalho foi a análise da influência do pH na formação de biofilmes de isolados vaginais de *C. albicans* e *C. glabrata*. Constatou-se que o pH afeta a biomassa, a estrutura e a composição da matriz de biofilmes de ambas as espécies; sendo a formação de biofilme de *C. glabrata* favorecida em condições ácidas e a de *C. albicans* em pH neutro. A análise da produção de matriz por biofilmes de *C. glabrata* em ambiente ácido foi aprofundada, tendo-se verificado que é regulada pelo Zap1. Este fator de transcrição bloqueia a secreção de 31 proteínas para a matriz, algumas potencialmente relacionadas com a deposição de carboidratos na matriz. Adicionalmente, o Zap1 induz a secreção de 134 proteínas, sugerindo um papel na modulação do perfil proteómico da matriz por condições ácidas, no qual foram encontradas 397 proteínas, reportadas pela primeira vez na matriz.

A influência das hormonas, progesterona e β-estradiol, em isolados vaginais de *C. albicans* e *C. glabrata*, foi também analisada, em condições ácidas. As hormonas levaram ao aumento de resistência das células ao peróxido de hidrogênio e a azóis, e afetaram a formação de biofilme de *C. albicans*, mas não de *C. glabrata*. Consistentemente, a análise transcriptómica de biofilmes de *C. albicans* formados na presença de progesterona levou à identificação de 166 genes reprimidos, incluindo o regulador de biofilme Tec1 e muitos de seus genes alvo, e também 54 genes induzidos, incluindo as bombas de efluxo Cdr1 e Cdr2. O perfil transcriptómico de biofilmes de *C. albicans* em condições ácidas, foi também analisado na ausência de hormona, tendo sido identificados 286 genes não previamente associados à formação de biofilme, e o Sfl1 como potencial regulador de biofilme em condições ácidas.

No geral, este estudo mostra que as condições vaginais modulam fatores de virulência de *Candida* de forma dependente da espécie, tendo sido revelados potenciais determinantes de virulência vaginal, contribuindo assim para um melhor conhecimento da patogenicidade vaginal de *Candida*.

Palavras-chave: candidíase vulvovaginal, determinantes de virulência vaginal, espécies de *Candida*.

Index

List of figures xii

List of tables xiv

Nomenclature xvi

Scientific outputs xix

Structure of the thesis and objectives xxi

Chapter 1 - Literature review 1

1.1 Introduction 3

1.2 Epidemiology of VVC 4

1.3 Microbiology of VVC 6

1.4 *Candida* virulence factors 8

1.4.1 Adhesion 9

1.4.2 Biofilm formation 10

1.4.3 Extracellular hydrolytic enzyme production 12

1.4.4 Filamentous forms development 13

1.4.5 Phenotypic switching 14

1.5 Risk factors for VVC 15

1.5.1 Host factors 16

1.5.1.1 Pregnancy and hormone replacement therapy 16

1.5.1.2 Antibiotics 20

1.5.1.3 Immunosuppression 23

1.5.1.4 Uncontrolled diabetes and glucocorticoids 27

1.5.1.5 Genetic predispositions 29

1.5.2 Behavioural factors 30

1.5.2.1 Contraceptives (oral pills, intrauterine devices and spermicides) 30

1.5.2.2 Sexual, clothing and hygienic habits 32

References 34

Chapter 2 - Biofilm formation of *Candida* vaginal isolates at acidic versus neutral pH 49

2.1 Introduction 51

2.2 Materials and Methods 52

2.2.1 *Candida* strains and initial culture conditions....52
2.2.2 Analysis of planktonic growth....52
2.2.3 Biofilm formation....53
2.2.4 Biofilm biomass quantification....53
2.2.5 Determination of biofilm cells' cultivability....53
2.2.6 Biofilm structure and thickness analysis....54
2.2.7 Analysis of biofilm cells' filamentous forms....54
2.2.8 Biofilm matrix extraction and analysis....54
2.2.8.1 Protein quantification and analysis....55
2.2.8.2 Carbohydrate quantification....56
2.2.8.3 (1,3)-β-D-glucan quantification....56
2.2.9 Statistical analysis....56
2.3 Results....57
2.3.1 *Candida albicans* and *C. glabrata* planktonic growth at acidic and neutral conditions....57
2.3.2 *Candida albicans* and *C. glabrata* biofilm formation at acidic and neutral conditions....57
2.3.3 Influence of pH on *C. albicans* filamentation....61
2.3.4 Influence of pH on *C. albicans* and *C. glabrata* biofilm matrix....62
2.4 Discussion....65
References....68

Chapter 3 - Biofilm matrix regulation by *Candida glabrata* Zap1 under acidic conditions 71
3.1 Introduction....73
3.2 Materials and Methods....74
3.2.1 Construction of mutant strains....74
3.2.1.1 Assembly of the deletion cassette....74
3.2.1.2 Transformation of *C. glabrata* cells....76
3.2.1.3 Construction of the complemented strain....77
3.2.2 Mutant strains' analysis....78
3.2.2.1 Initial culture conditions....78
3.2.2.2 Planktonic growth analysis....78

3.2.2.3 Biomass formation and analysis 78
3.2.2.4 Planktonic supernatant and biofilm matrix analysis 79
3.2.2.5 Susceptibility assays 79
3.2.2.6 Measurement of *ZAP1* transcript by quantitative real-time PCR 80
3.2.2.7 Statistical analysis 81
3.3 Results 81
3.3.1 Mutant strains' construction 81
3.3.2 Influence of *ZAP1* deletion on *C. glabrata* planktonic growth and biofilm formation 82
3.3.3 Role of *C. glabrata* Zap1 in biofilm matrix production 85
3.3.4 Regulation of biofilm matrix proteins by *C. glabrata* Zap1 87
3.4 Discussion 91
References 95

Chapter 4 - Influence of hormones on biofilm formation and resistance of *Candida* vaginal isolates 97
4.1 Introduction 99
4.2 Materials and Methods 100
4.2.1 Strains and initial culture conditions 100
4.2.2 Hormones 100
4.2.3 Planktonic growth analysis 101
4.2.4 Biofilm formation and analysis 101
4.2.4.1 Biomass and biofilm cells cultivability analysis 101
4.2.4.2 Filamentous forms analysis 102
4.2.5 Susceptibility tests 102
4.2.5.1 Azoles testing 102
4.2.5.2 Hydrogen peroxide testing 103
4.2.6 Statistical analysis 103
4.3 Results 104
4.3.1 Hormones influence on *Candida* planktonic growth 104
4.3.2 Hormones modulation of *Candida* biofilm formation 105
4.3.3 Hormones effect on *C. albicans* filamentous forms development 106

4.3.4 Hormones influence on *Candida* cells resistance 108
4.3.4.1 Susceptibility to azoles 108
4.3.4.2 Susceptibility to hydrogen peroxide 109
4.4 Discussion 111
References 116

Chapter 5 - Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: a transcriptomic analysis 119
5.1 Introduction 121
5.2 Materials and Methods 122
5.2.1 Strains and initial culture conditions 122
5.2.2 Planktonic growth analysis 122
5.2.3 Biofilm formation and analysis 123
5.2.3.1 Biomass and biofilm cells' cultivability analysis 123
5.2.3.2 Metabolic activity 123
5.2.3.3 Biofilm structure 124
5.2.3.4 Biofilm matrix extraction and analysis 124
5.2.3.5 Biofilm cells' susceptibility testing 124
5.2.4 Transcriptomic analysis 125
5.2.4.1 RNA extraction 125
5.2.4.2 Microarrays analysis 126
5.2.5 Measurement of gene transcript based on quantitative real-time PCR 127
5.2.6 Experiments using *C. albicans* mutant strains 127
5.2.7 Statistical analysis 127
5.3 Results and discussion 128
5.3.1 Effect of progesterone on planktonic growth and biofilm formation 128
5.3.2 Effect of progesterone on biofilm matrix composition 129
5.3.3 Transcriptional profiling of *C. albicans* cells present in biofilms formed in the presence or absence of progesterone 130
5.3.3.1 Transcriptome wide-alterations in *C. albicans* biofilms in the absence of progesterone 131

5.3.3.2 Transcriptome wide-alterations in *C. albicans* biofilms in the presence of progesterone 137
References 144

Chapter 6 - General conclusions and work perspectives 147
6.1. General conclusions 148
6.2. Work perspectives 150

Annex I 152
Annex II 161
Annex III 162

List of figures

Chapter 1 – Literature review

Figure 1.1 Reconstituted human vaginal epithelium (RHVE) infected with *Candida albicans*, *Candida glabrata* and co-infected with *C. albicans and C. glabrata* 6

Figure 1.2 Host-related and behavioural risk factors for vulvovaginal candidiasis and respective effects.. 15

Figure 1.3 Scanning electron microscopy images of *Candida albicans* ATCC biofilms formed in the absence and presence of 2 μM of progesterone. 19

Figure 1.4 Scanning electron microscopy images of *Candida tropicalis* ATCC biofilms formed at pH 4, pH 7 and pH 8. 23

Chapter 2 – Biofilm formation of *Candida* vaginal isolates at acidic versus neutral pH

Figure 2.1 *Candida albicans* and *Candida glabrata* planktonic growth at pH 4 and pH 7. 57

Figure 2.2 *Candida albicans* and *Candida glabrata* biofilm formation at pH 4 and pH 7 58

Figure 2.3 Effect of pH on *Candida albicans* biofilm structure and thickness 59

Figure 2.4 Effect of pH on *Candida glabrata* biofilm structure and thickness.....60

Figure 2.5 Effect of pH on *Candida albicans* filamentous forms development 62

Figure 2.6 Influence of pH on *Candida glabrata* biofilm matrix proteins 64

Chapter 3 – Biofilm matrix regulation by *Candida glabrata* Zap1 under acidic conditions

Figure 3.1 Assembly of the deletion cassette 76

Figure 3.2 Insertion of *SAT1* flipper cassette into *Candida glabrata* genome 77

Figure 3.3 Fragments' size obtained after the amplification of *Candida glabrata ZAP1* gene. 82

Figure 3.4 Influence of *ZAP1* deletion on *Candida glabrata* planktonic growth.....82

Figure 3.5 Effect of *ZAP1* deletion on *Candida glabrata* biofilm formation 83

Figure 3.6 Effect of *ZAP*1 deletion on *Candida glabrata* biofilm structure 84

Figure 3.7 Expression of *ZAP1* gene in *Candida glabrata* planktonic and biofilm cells 85

Figure 3.8 Functional distribution of *Candida glabrata* biofilm matrix proteins at acidic conditions.....87

Figure 3.9 Regulation of biofilm matrix proteins by *Candida glabrata* Zap1 90

Chapter 4 – Influence of hormones on biofilm formation and resistance of *Candida* vaginal isolates

Figure 4.1 Effect of hormones on *Candida albicans* and *Candida glabrata* planktonic growth 104

Figure 4.2 Effect of hormones on *Candida albicans* and *Candida glabrata* biofilm formation 105

Figure 4.3 Effect of hormones on *Candida albicans* filamentous forms development 107

Chapter 5 – Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: a transcriptomic analysis

Figure 5.1 Effect of progesterone on *Candida albicans* biofilm formation 129

Figure 5.2 Functional distribution of genes found to be biofilm-regulated in *Candida albicans* cells cultivated for 24 h in RPMI at pH 4 134

Figure 5.3 Proposed transcriptional regulatory network underlying the control of *Candida albicans* biofilm cells after 24h of cultivation in RPMI at pH 4.....135

Figure 5.4 Sfl1 is required for maximal *Candida albicans* biofilm formation under acidic conditions 136

Figure 5.5 Venn diagram showing the number of *Candida albicans* genes whose transcription was found to be altered (up- or down-regulated) in biofilms formed with and/or without progesterone.....138

Figure 5.6 Schematic representation of the proposed genetic regulation of *Candida albicans* biofilm cells by progesterone 141

Figure 5.7 Tec1-documented targets that were found to have a reduced expression in progesterone-exposed biofilm cells..... 143

Annex II

Figure AII.1 Progesterone effect on *Candida albicans* planktonic cells 161

Figure AII.2 Transcript levels, estimated by qRT-PCR, of *Candida albicans TEC1*, *CDR1* and *CZR2* genes 161

List of tables

Chapter 1 – Literature review

Table 1.1 Epidemiologic studies concerning the VVC incidence in symptomatic women and incidence of vaginal colonization by *Candida* species in asymptomatic women 5

Table 1.2 Epidemiologic studies concerning the distribution of *Candida* species in women with VVC ...7

Table 1.3 Incidence of VVC in pregnant and non-pregnant women 16

Table 1.4 Incidence of VVC in women who used or not antibiotics and incidence of antibiotic use in women with and without VVC 20

Chapter 2 – Biofilm formation of *Candida* vaginal isolates at acidic versus neutral pH

Table 2.1 Influence of pH on *Candida albicans* and *Candida glabrata* biofilm matrix production 63

Chapter 3 – Biofilm matrix regulation by *Candida glabrata* Zap1 under acidic conditions

Table 3.1 Influence of *ZAP1* deletion on *Candida glabrata* secreted components 86

Table 3.2 Influence of *ZAP1* deletion on *Candida glabrata* biofilm susceptibility to caspofungin. 86

Chapter 4 – Influence of hormones on biofilm formation and resistance of *Candida* vaginal isolates

Table 4.1 Susceptibility of *Candida albicans* and *Candida glabrata* to fluconazole and voriconazole in presence of progesterone and/or β-estradiol 109

Table 4.2 Susceptibility of *Candida albicans* and *Candida glabrata* to hydrogen peroxide in presence of progesterone and/or β-estradiol. 110

Chapter 5 – Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: a transcriptomic analysis

Table 5.1 *Candida albicans* strains used in this study.....122

Table 5.2 Progesterone effect on *Candida albicans* biofilm matrix composition. 130

Table 5.3 Subset of genes found to be biofilm-regulated (up- and down-) in *Candida albicans* cells cultivated 24 h at pH 4 132

Table 5.4 Subset of progesterone-responsive genes in *Candida albicans* SC5314 biofilms grown 24 h at pH 4..... 139

Annex I

Table AI.1 Primers used in the study of Chapter 3 152

Table AI.2 List of *Candida glabrata* biofilm matrix proteins at acidic conditions 153

Table AI.3 List of *Candida glabrata* biofilm matrix proteins found to be regulated by Zap1 160

Annex III

Table AIII.1 Forward (FW) and reverse (RV) primers used for qRT-PCR of Chapter 5 162

Table AIII.2 List of genes found to be biofilm-regulated (up- and down) in *Candida albicans* cells cultivated 24 h at pH 4. 163

Table AIII.3 Regulatory associations between biofilm-induced genes and biofilm-related transcriptions factors..... 181

Table AIII.4 List of genes found to be altered (up- and down-regulated) in *Candida albicans* biofilms grown 24 h in RMPI supplemented with progesterone 186

Table AIII.5 List of progesterone-responsive genes in *Candida albicans* SC5314 biofilms grown 24 h at pH4..... 205

Nomenclature

ABC	ATP-binding cassette
ALS	Agglutinin-like sequence
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
BV	Bacterial vaginosis
CAA	Carboxylic acid amide
cAMP	Cyclic adenosine monophosphate
CBP	Corticosteroid-binding protein
cDNA	Complementary DNA
CFU	Colony forming units
CGD	*Candida* genome database
CLSI	Clinical and laboratory standards institute
CLSM	Confocal laser scanning microscope
Ct	Cycle threshold
CV	Crystal violet
DC	Dendritic cells
DM	Diabetes mellitus
DMPA	Depot medroxyprogesterone acetate
DMSO	Dimethyl-sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBP	Estrogen-binding protein
EDTA	Ethylenediamine tetraacetic acid

Epa	Epithelial adhesin
FDR	Protein-level false discovery rate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GPI	Glycosylphosphatidylinositol
HIV	Human immunodeficiency virus
HRT	Hormone replacement therapy
Hwp	Hyphal wall protein
IAA	Indole-3-acetic acid
IL	Interleukin
ITS	Internal transcribed spacer
IUDs	Intrauterine devices
LB	Lysogeny Broth
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LR	Lectin-like receptor
MBL	Mannose binding lectin
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
MFC	Minimumfungicidal concentration
MFS	Major facilitator superfamily
MOPS	3-(N-Morpholino) propanesulfonicacid
mRNA	Messenger RNA
N9	Nonoxynol-9
NADP	Nicotinamide adenine dinucleotide phosphate
NCAC	non-*Candida albicans Candida*
NO	Nitric oxide
NRT	Non-reverse transcriptase
OCP	Oral contraceptive pills
PAMPs	Pathogen associated molecular patterns
PBL	Peripheral blood lymphocyte

PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PMS	Phenazine methosulfate
qRT-PCR	Quantitative real-time PCR
rev	Revolutions
RHVE	Reconstituted human vaginal epithelium
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RVVC	Recurrent VVC
Sap	Secreted aspartyl proteinase
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	Scanning electron microscopy
SLPI	Secretory leukocyte protease inhibitor
SOC	Super optimal broth with catabolite repression
TCEP	Tris(2-carboxyethyl)phosphine)
TE	Tris-EDTA
TEAB	Triethylamonium bicarbonate
TFA	Trifluoroacetic acid
TLR	Toll-like receptor
UDP	Uridine diphosphate
VEC	Vaginal epithelial cells
VVC	Vulvovaginal candidiasis
XTT	2,3-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazoliumhydroxide
YPD	Yeast peptone dextrose
YPM	Yeast peptone maltose

Scientific outputs

Papers in peer reviewed journals:

Gonçalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S. Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. *Critical Reviewsin Microbiology* (2016); 42(6):905–27.Doi: 10.3109/1040841X.2015.1091805. **(Chapter 1)**

Gonçalves B, Fernandes L, Henriques M, Silva S. Biofilm formation of *Candida* vaginal isolates at acidic versus neutral pH (submitted). **(Chapter 2)**

Gonçalves B, Pires DP, Fernandes L, Azevedo NM, Henriques M, Silva S. Biofilm Matrix regulation by *Candida glabrata* Zap1 under acidic conditions (submitted). **(Chapter 3)**

Gonçalves B, Azevedo NM, Henriques M, Silva S. Hormones modulate *Candida* vaginal isolates biofilm formation and decrease their susceptibility to azoles and hydrogen peroxide. *Medical Mycology* (2019). Doi: 10.1093/mmy/myz070 (in press). **(Chapter 4)**

Gonçalves B, Bernardo R, Wang C, Pedro NA, Butler G, Azeredo J, Henriques M, Mira NP, Silva S. Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: a transcriptomic analysis. *International Journal of Medical Microbiology* (2019) (in review). **(Chapter 5)**

Ferreira C, **Gonçalves B**, Vilas Boas D, Oliveira H, Henriques M, Azeredo J, Silva S. *Candida tropicalis* biofilm and human epithelium invasion is highly influenced by environmental pH. *Pathogens and Disease* (2016); 74(8):ftw101. Doi: 10.1093/femspd/ftw101.

Rodrigues CF, **Gonçalves B**, Rodrigues ME, Silva S, Azeredo J, Henriques M. The effectiveness of voriconazole in therapy of *Candida glabrata's* biofilms oral infections and its influence on the matrix composition and gene expression. *Mycopathologia* (2017). Doi: 10.1007/s11046-017-0135-7.

Posters in conferences:

Gonçalves B, Bernardo R, Wang C, Butler G, Azeredo J, Henriques M, Mira NP, Silva S. *Candida albicans* vulvovaginal biofilm response to progesterone: genes involved. Biofilms 7, Porto, Portugal, 26-28 June, 2016.

Gonçalves B, Bernardo R, Wang C, Pedro NA, Butler G, Azeredo J, Henriques M, Mira NP, Silva S. Progesterone modulates *Candida albicans* biofilm formation and gene expression. EUROBIOFILMS 2017 - 5th European Congress on Microbial Biofilms, Amsterdam, Netherlands, 19-22 September, 2017.

Gonçalves B, Azevedo NM, Fernandes L, Henriques M, Silva S. Biofilm formation by *Candida albicans* and *Candida glabrata* under acidic conditions: implications on vulvovaginal candidiasis. 6th International Conference on Mycology and Fungal Infections, Madrid, Spain, 7-8 October, 2019.

Rodrigues CF, **Gonçalves B**, Rodrigues ME, Silva S, Azeredo J, Henriques M. Fluconazole versus voriconazole *Candida glabrata's* biofilms response to different azoles 7th Trends in Medical Mycology, Lisbon, Portugal, 2015.

Ferreira C, **Gonçalves B**, Vilas Boas D, Oliveira H, Henriques M, Azeredo J, Silva S. *Candida tropicalis* biofilm is highly influenced by the environmental human pH. Biofilms 7, Porto, Portugal, 26-28 June, 2016.

Objectives and structure of the thesis

Several risk factors can disturb the balance between *Candida* vaginal colonization and the host environment, promoting VVC development, to which a number of *Candida* virulence factors contribute, including the biofilm formation on vaginal mucosal surfaces or intrauterine devices (IUDs). Despite a growing list of recognized risk factors, the mechanisms underlying the transition of *Candida* species from commensal into pathogenic state are still unclear. This transition is associated with the expression of genes and production/secretion of their corresponding proteins, which are essential for the initiation and progression of the disease. Despite the high incidence of VVC and increasing difficulty of its treatment there are only few studies concerning *Candida* vaginal virulence. Thus, it is of major importance to disclose relevant vaginal virulence determinants and their species-specificity, because the biology and virulence of *Candida* species vary considerably. In this sense, the main goal of this investigation was to assess the role of vaginal conditions in the modulation of relevant virulence factors of the most common species in vaginal tract, *C. albicans* and *C. glabrata*, and identification of potential vaginal virulence determinants, using advanced and prominent techniques of genomic, transcriptomic and proteomic approaches.

This thesis is divided into six chapters representing the various studies of the research conducted: the literature review (Chapter 1), the study of the modulation of *Candida* virulence factors by acidic conditions prompted by lactic acid (Chapter 2) and associated virulence determinants (Chapter 3) and the study of the modulation of *Candida* virulence features by hormones (Chapter 4) and associated virulence determinants (Chapter 5).

Chapter 1 is a literature review presenting the current knowledge on *Candida* vaginal virulence and VVC, including the epidemiology, microbiology and risk factors of the disease, and also virulence factors of *Candida* species potentially related with it.

In **Chapter 2** is presented the analysis of the role of vaginal acidic conditions, which are promoted by lactic acid, on the growth and virulence traits of *C. albicans* and *C. glabrata* vaginal isolates. A comparison of the growth curves, cells cultivability, biofilm biomass, biofilm structure and matrix composition obtained at pH 4 and pH 7, was performed for each species and strain. In *C. albicans* the influence of pH on the ability to form filamentous forms in the planktonic and biofilm states, was also evaluated.

The work of **Chapter 3** aimed to deep the knowledge on *C. glabrata* biofilm matrix composition and regulation at acidic conditions, as the matrix is a relevant but largely understudied feature of *C.*

glabrata biofilms. For that, the role of the transcription factor Zap1 on *C. glabrata* biofilm matrix regulation was investigated using a constructed knockout mutant strain. The phenotypic characterization of the mutant strain in planktonic and biofilm modes was performed and the role of Zap1 in biofilm matrix production was assessed, along with the identification of matrix proteins specifically regulated by Zap1. The secretome profile of *C. glabrata* biofilm was analysed using mass spectroscopy analysis, and its modulation by acidic conditions, as well as the potential role of Zap1 on this process, were also investigated.

The work of **Chapter 4** aimed to study the influence of the reproductive hormones, progesterone and β-estradiol, which have been shown to promote VVC, on virulence and resistance traits of *C. albicans* and *C. glabrata* vaginal isolates. The influence of hormones was evaluated on the planktonic growth and biofilm formation of both species, and also on *C. albicans* ability to form filamentous forms. Additionally, hormones' influence on the susceptibility of planktonic and biofilm cells to the antifungals, fluconazole and voriconazole, and to peroxide hydrogen, a natural vaginal barrier against *Candida* species, was assessed.

In **Chapter 5** the molecular mechanisms underlying the effect of progesterone, at pregnancy level, on *C. albicans* biofilm formation at acidic conditions were studied, using a high-throughput transcriptomic technique. The transcriptomic profiles of *C. albicans* biofilms formed in presence and absence of progesterone were analysed and compared, and genes specifically responsive to progesterone and acidic conditions were identified. Some putative virulence determinants identified were further studied using mutant strains to evaluate their potential role on biofilm formation and target gene regulation at vaginal conditions.

Chapter 6 comprises the major conclusions reached during this work and includes suggestions of additional work of interest in the scope of this thesis.

Overall, this investigation aimed to increase the knowledge on *C. albicans* and *C. glabrata* vaginal virulence and its underlying mechanisms, including the reveal of potential vaginal virulence determinants, which may contribute to the disclosure of new targets for the development of more effective therapies to treat VVC.

Chapter 1

Literature review

Main goal

To provide the current state of knowledge on *Candida* virulence factors associated with vaginal pathogenicity and on risk factors that predispose to vulvovaginal candidiasis, also including a revision of the epidemiology and microbiology of the disease.

This chapter is based on the following article:

Gonçalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S. Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. *Crit Rev Microbiol* (2016); 42(6):905–27.
Doi: 10.3109/1040841X.2015.1091805

1.1 Introduction

The species from genus *Candida* have a wide distribution in nature and can be found in humans, domestic and wild animals as well as in diverse environments including hospitals [1]. These yeasts belong to the normal flora of humans and can colonize the mucosal surfaces of the genital, urinary, respiratory and gastrointestinal tracts, as well as oral cavity, nails, scalp and skin [2]. However, *Candida* species can be commensal organisms or transform a symptomless colonization into an infection. Thus, these species are characterized as opportunistic and can change from harmless to pathogenic upon variation of the host conditions. *Candida* infections are mainly superficial, but in severely immunocompromised patients serious systemic infections can occur [3]. Most, if not all women carry *Candida* in the vagina at some point of their lives, with or without symptoms of infection [4]. *Candida* organisms gain access to the lower genital tract mainly from the adjacent perianal area [5]. There is a balance between *Candida* organisms and vaginal defence mechanisms against *Candida*, such as lactobacilli and immune responses that allow the persistence of *Candida* species as vaginal commensals [6]. Thereby, changes in the host vaginal/vulvar environment can lead to a *Candida* infection, named vulvovaginal candidiasis (VVC). VVC is considered when there are signs and symptoms of inflammation in the presence of *Candida* species but in the absence of other infectious agents [7]. VVC can be classified into uncomplicated and complicated cases, a classification that has been adapted over the years. Uncomplicated VVC is characterized by fewer than four episodes per year with mild to moderate severity caused by *Candida albicans* in apparently healthy women. Complicated VVC include episodes due to non-*Candida albicans Candida* (NCAC) species or severe cases caused by any *Candida* species. Moreover, recurrent VVC (RVVC), which is characterized by four or more episodes per year, and VVC in presence of recognized risk factors (e.g. pregnancy, diabetes and immunosuppression) are also classified as complicated VVC [7–9].

The clinical symptoms of VVC are nonspecific and can be associated with a variety of other vaginal diseases, such as bacterial vaginosis, trichomoniasis and gonorrhoea [10]. The most common clinical manifestations are vulvar pruritus and burning accompanied by vaginal soreness and irritation leading to dyspareunia and dysuria. Vulvar and vaginal erythema, edema and fissures are also commonly found [11]. Topical antimycotic agents (e.g. creams, lotions and vaginal suppositories) and oral therapy are available for VVC. Topical polyenes (e.g nystantin) achieve a mycological cure rate of about 75-80% and topical azoles (e.g. clotrimazole, miconazole, econazole, fenticonazole) of about 85-90% [12], in uncomplicated cases. Oral azole agents (fluconazole and itraconazole) achieve comparable cure rates than do topical agents [12,13] and a study in Portugal reported that most women prefer the

convenience of oral administration [14]. However, oral azoles have a potential side effect of toxicity and are not recommended in pregnant women [12]. In addition, topical and oral azoles are poorly effective in VVC caused by *Candida glabrata* in comparison to *C. albicans* [15,16]. In fact, complicated infections such as those caused by NCAC species or in presence of risks factors are much more difficult to treat requiring intensive regimens [12]. Prevention treatments such as topical vaginal use of recombinant mannose binding lectin [17] and anti-*Candida* vaccines [18,19] have been investigated as possible preventions for VVC.

VVC affects millions of women every year and has been considered an important public health problem. Although not associated with mortality, the morbidity associated with VVC makes it a major cause of mental distress, causing pain, great discomfort, altered self-esteem, anxiety and, impairing work performance, also interfering with sexual and affective relations [20]. Nevertheless, in severely immunocompromised hosts the infection may disseminate to the blood stream leading to invasive candidiasis, which is associated with high mortality rate [3]. VVC is also associated with considerable direct and indirect economic costs [21] and enhanced susceptibility to human immunodeficiency virus (HIV) infection [22]. Especially when VVC is not promptly treated, many complications have been appointed as its consequence, such as pelvic inflammatory disease, infertility, ectopic pregnancy, pelvic abscess, spontaneous abortion and menstrual disorders. Therefore, prevention, early diagnosis and prompt treatment of VVC, especially among risk groups, are essential to avert the complications [23].

Despite therapeutic advances VVC remains a common disease, for which triggering factors and their underlying mechanisms are not fully understood. This Chapter provides information about the current state of knowledge on the risk factors predisposing to VVC, also including a revision of the epidemiology and microbiology of VVC, as well as of *Candida* virulence factors associated with vaginal pathogenicity.

1.2 Epidemiology of VVC

Vulvovaginal candidiasis is not a reportable disease and therefore the information on its incidence is incomplete and based on epidemiology studies that are often hampered by inaccuracies of diagnosis and/or the use of non-representative populations [20]. VVC is considered the second most common cause of vaginitis after bacterial vaginosis [10]. It is estimated that approximately 10-15% of asymptomatic women are colonized with *Candida*, 70-75% of women will experience an episode of VVC

in their lifetimes, 50% of initially infected women will suffer a second VVC event and 5-10% of all women will develop RVVC [20].

Symptoms and signs of VVC are not specific to the disease and the presence of *Candida* in the vagina is not necessarily indicative of VVC since asymptomatic women can be colonized [10]. Therefore, the diagnosis of VVC requires correlation of clinical findings and laboratory confirmation of *Candida*. Table 1.1 highlights the more relevant epidemiologic studies published over the last years concerning the incidence of VVC, diagnosed in symptomatic women with laboratory confirmation. The incidence of vaginal colonization by *Candida* species is also shown, whenever asymptomatic women were included.

Table 1.1 Epidemiologic studies concerning the VVC incidence in symptomatic women and incidence of vaginal colonization by *Candida* species in asymptomatic women

Country	Symptomatic women		Asymptomatic women		Reference
	Number	With VVC (%)	Number	Colonized (%)	
Nigeria	83	16.9	117	11.9	[24]
Egypt	381	40.9	-	-	[25]
Tunisia	481	48.0	-	-	[26]
Tunisia	2083	30.3	-	-	[27]
Iran	310	51.6	-	-	[28]
Iran	234	28.2	-	-	[29]
Iran	120	28.3	-	-	[30]
Bangladesh	183	31.2	-	-	[31]
India	150	24.0	-	-	[32]
India	160	35.0	-	-	[33]
India	601	18.5	-	-	[34]
India	1050	20.4	-	-	[35]
India	300	17.7	-	-	[36]
Nepal	193	25.3	-	-	[37]
China	301	61.8	-	-	[38]
Brazil	23	43.5	112	14.3	[39]
Brazil	121	47.9	165	17.0	[40]
Jamaica	422	29.6	-	-	[41]
Italy	13014	19.5	11551	11.6	[42]
Austria	10463	30.5	-	-	[43]
Greece	4743	12.1	-	-	[44]
Turkey	569	42.2	-	-	[45]
Israel	208	35.5	100	15.0	[46]
Australia	342	42.7	-	-	[47]

The incidence of VVC in symptomatic women varies depending on the locations as well as the populations studied. The studies published during the last years reported incidences of the disease in symptomatic women that range from 12.1 to 61.8% (Table 1.1). The highest incidences were reported by epidemiologic studies assessed in some Asian countries (China [38] and Iran [28], with 61.8% and 51.6% respectively), followed by Tunisian [26] and Brazilian [39,40] studies. The lowest incidences were reported in European countries (Greece [44] and Italy [42], with 12.1 and 19.5 % respectively) and India [32,34–36]. All epidemiologic studies are consensual in reporting higher incidence of VVC in women at reproductive age (20-40 years) than those at menopause. Regarding asymptomatic colonization, Brazilian [39,40], Italian [42], Israeli [46] and Nigerian [24] studies reported vaginal colonization by *Candida* species in asymptomatic women with a prevalence between 11.6 and 17.0% (Table 1.1). VVC affects women globally and therefore, further large studies from diverse countries are needed to a better knowledge of the incidence of VVC and vaginal asymptomatic colonization by *Candida* species.

1.3 Microbiology of VVC

The most common *Candida* species associated with VVC are *C. albicans*, *C. glabrata*, *Candida tropicalis*, *Candida parapsilosis* and *Candida krusei*. Typically, a single species is identified, but two or more species have been found in the vaginal discharges of women with VVC (1 to 10%), mainly an association between *C. albicans* and *C. glabrata* [26,28,29,45,48]. In fact, *C. albicans* is the most common species identified in women with VVC followed by *C. glabrata* (Table 1.2). An *ex vivo* study demonstrated higher colonization and invasion of vaginal tissue by *C. albicans* than by *C. glabrata*, but an enhanced invasion by *C. glabrata* in co-infection with *C. albicans* (Figure 1.1) [49].

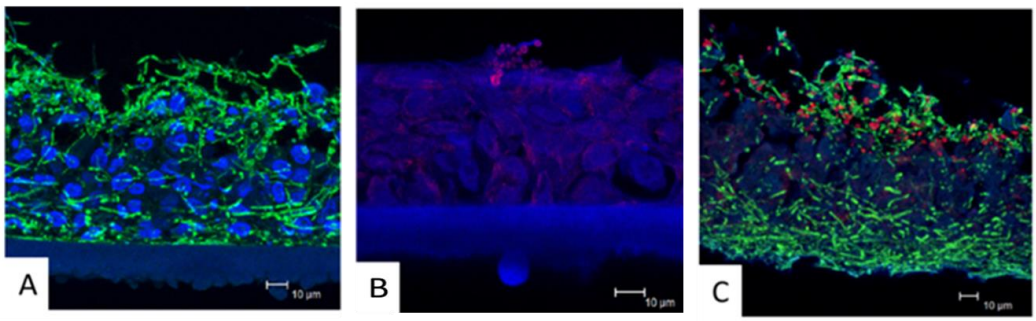


Figure 1.1 Reconstituted human vaginal epithelium (RHVE) infected with **(A)** *Candida albicans* (in green), **(B)** *Candida glabrata* (in red) and **(C)** co-infected with *C. albicans and C. glabrata* [49].

Table 1.2 highlights relevant epidemiologic studies published over the last years concerning the distribution of the most common vaginal *Candida* species identified in women with VVC.

Table 1.2 Epidemiologic studies concerning the distribution of *Candida* species in women with VVC.

Country	No. of subjects	Incidence of *Candida* species (%)					Ref.
		C. albicans	***C. glabrata***	***C. tropicalis***	***C. parapsilosis***	***C. krusei***	
USA	429	75.7	15.6	1.1	3.8	3.8	[50]
Jamaica	125	83.2	4.8	11.2	-	-	[41]
Brazil	40	70.0	20.0	7.5	-	-	[51]
Brazil	71	67.6	11.2	1.4	2.8	-	[52]
Italy	909	77.1	14.6	2.3	0.6	4.0	[53]
Slovakia	227	87.7	6.2	0.9	0.9	2.2	[54]
Austria	3243	87.9	3.4	0.2	0.5	0.3	[43]
Greece	576	80.2	7.3	1.6	2.1	1.0	[44]
Spain	145	86.7	11.0	0.6	1.4	-	[55]
Turkey	240	44.2	29.6	-	-	5.8	[45]
India	111	35.1	50.4	10.8	0.9	2.7	[34]
India	215	46.9	36.7	2.8	10.2	1.4	[35]
India	56	44.0	11.0	36.0	7.0	2.0	[33]
China	1070	90.4	7.9	0.9	0.6	0.1	[56]
China	186	91.4	4.3	3.2	1.1	-	[38]
Iran	175	65.1	13.1	6.2	0.6	4.0	[48]
Iran	160	86.8	3.8	-	-	3.1	[28]
Tunisia	295	76.3	19.3	1.4	-	-	[26]
Tunisia	710	76.6	18.2	1.4		1.5	[27]
Nigeria	517	20.3	33.8	17.9	4.3	-	[57]
Australia	1221	89.0	7.3	0.7	1.0	1.0	[58]

Many epidemiologic studies report higher association of VVC with *C. albicans* than non-*Candida albicans Candida* (NCAC) species (Table 1.2). In American [41,50–52], European [43,44,53–55] and Australian [58] studies *C. albicans* was the most common species identified in women with the disease (67.6 to 89.0%), followed by *C. glabrata* (3.4 to 20.0%). Also Chinese [38,56], Tunisian [26,27] and Iranian [28,48] studies show a predominance of *C. albicans* (65.1 to 91.4%), however in some Asian and African studies (Turkey [45], India [33–35] and Nigeria [57]), NCAC species appear to be more commonly associated with VVC, especially *C. glabrata* (29.5 to 50.4%) (Table 1.2). *Candida tropicalis* is the second most common NCAC species, with the higher percentages identified in India (36.0%) [33],

Nigeria (17.9%) [57] and Jamaica (11.2%) [41]. Although *Candida* species presented in Table 1.2 are the most commonly found in the vaginal tract, some recent studies have reported incidence of other species, including *Candida kefyr, Candida dubliniensis*, and *Candida guilliermondii* [28–30].

Historically, 85-95% of *Candida* species identified in women with VVC were *C. albicans* [59–63], however most studies published during the last years reported incidence of *C. albicans* below 85% and in some countries even below 50% (Table 1.2). It has been suggested that the widespread and inappropriate use of antifungal treatments (self-medication and prolonged antifungal therapy) may lead to the selection of NCAC species (such as *C. glabrata*), which are more resistant to the commonly used antifungal agents than *C. albicans* [50,64]. In fact, NCAC species have been more commonly isolated among patients with RVVC than in women with sporadic VVC [26,44,50,58], possibly due to a higher antifungal exposure and widespread use of over-the-counter antimycotics among patients with RVVC [65]. High percentages of NCAC species causing VVC, mainly *C. glabrata*, have been also associated with increasing age [56,58,66], patients with uncontrolled diabetes [67,68] and HIV-infected women [53,63]. These associations are possibly due to changes in patient physiology, hormone balance and decrease in immune functions.

Compared to *C. albicans*, NCAC species are generally associated with higher resistance to the azoles [50,64], the most commonly prescribed class of antifungal agents. The use of non-azole antifungals, such as boric acid and flucytosine, may be effective in treating VVC caused by NCAC species, especially *C. glabrata* [69], which demonstrates intrinsically low susceptibility to the azoles and the ability to develop high resistance to them [50,64,70]. Additionally, it has been reported that azoles have reduced *in vitro* potency at vaginal acidic pH, being *C. glabrata* more affected than *C. albicans* [71]. The high resistance levels of NCAC species to the commonly used treatments associated to an increasing identification of these species in women with VVC highlights the importance of identifying *Candida* species within vaginal samples, in order to provide physicians with information concerning the proper treatment for their patients.

1.4 *Candida* virulence factors

Until a few decades ago it was believed that *Candida* microorganisms passively participated in the establishment of an opportunistic fungal infection, caused only by an organic weakness or an immunocompromised host. Today, there is consensus that these yeasts actively participate in the pathogenesis of the disease process, using mechanisms of aggression called virulence factors [72].

Thus, the pathogenicity of *Candida* species is mediated by a number of virulence factors that include adhesion, biofilm formation, extracellular hydrolytic enzyme production, filamentous forms development and phenotypic switching.

1.4.1 Adhesion

Adhesion of *Candida* to host surfaces is required for initial colonization of human tissues, contributes to persistence of the microorganism within the host and is essential in the establishment of infection [73]. Therefore, the primary event in VVC is the adhesion of *Candida* species to vaginal epithelial cells. Some studies have confirmed that *Candida* species have the ability to adhere to this type of cells [74,75]. In addition, *Candida* species can adhere to the surface of medical devices, often promoting device-related infections, like VVC in women using intrauterine device as contraceptive method [76]. The initial attachment of *Candida* cells to biotic and abiotic surfaces is mediated by cell surface physicochemical properties and promoted by specific cell surface proteins, called adhesins [77]. Adhesins recognize host ligands such as serum proteins and components of the extracellular matrix of host tissues (e.g. laminin, fibronectin, collagen, vitronectin and entactin), or promote the binding to abiotic surfaces through hydrophobic interactions [78]. In *C. albicans* a major group of adhesins is encoded by the agglutinin-like sequence (*ALS*) gene family which comprises eight members (*ALS1-7, 9*) [79]. Cheng et al. [80] detected expression of all *ALS* genes in vaginal specimens of women with VVC, but expressions of *ALS1*, *ALS2*, *ALS3* and *ALS9* were detected more frequently than of *ALS4* to *ALS7*. These investigators also demonstrated that the expression of *C. albicans ALS* genes has host-site specific influences. Rahimi et al. [81] also reported expression of *ALS2* and *ALS9* genes in several clinical isolates of women with VVC and suggested that these genes play a critical role in vaginal infections. In *C. glabrata*, up to 23 different genes encoding epithelial adhesins (*EPA* gene family) were already identified, and three genes, *EPA1*, *EPA6* and *EPA7*, have been shown to encode functional adhesins [82]. Alves et al. [49] detected expression of these three genes in reconstituted human vaginal epithelium (RHVE) infected with *C. glabrata*, with the highest value exhibited by *EPA1*. These investigators also detected all *ALS* genes in RHVE infected with *C. albicans,* with the highest expression exhibited by *ALS3* and *ALS6* and the lowest by *ALS7*. Interestingly, in RHVE co-infected with *C. glabrata* and *C. albicans* the *EPA* genes were down-regulated or absent and *ALS* genes were generally similar to those observed in single infection, with the exception of a highly increase in *ALS3* expression. These results suggest that probably *ALS3* but not *EPA* adhesins are associated with the enhanced RHVE

invasion by *C. glabrata* observed in the presence of *C. albicans*. In *C. parapsilosis* were identified eleven genes for putative cell wall adhesins-like proteins [83] and at least three Als proteins were identified in *C. tropicalis* and *C. dubliniensis* [84]. However, in most NCAC species the function of identified adhesins was still poorly studied.

1.4.2 Biofilm formation

Adhesion of *Candida* cells to host epithelium or medical devices has been implicated as an early step in biofilm formation. Biofilms are structured communities of microorganisms, irreversibly attached to a surface, with a high degree of organization and a self-produced extracellular matrix [85]. Biofilms are the most prevalent growth form of microorganisms in nature, with up to 80% of all microorganisms, in the environment, existing in biofilm communities. It is also suggested that over 65% of all human infections are related to microbial biofilms [86]. Biofilm formation is an important virulence factor for *Candida* species as it confers unique phenotypic characteristics compared to their planktonic counterpart cells including significant resistance to antifungal agents, host defence mechanisms and physical and chemical stress [86]. Furthermore, biofilm cells exhibit metabolic cooperation, community-based regulation of gene expression and the ability to withstand the competitive pressure from other organisms [87,88]. The association of microorganisms into biofilms is a form of protection for their development and contributes to their survival in hostile environmental conditions [87].

Clinically, the most important phenotype of biofilms is their extraordinary resistance to the conventional antifungal therapy, which has been reported to be up to 1000-fold higher than of their planktonic counterpart cells [88]. However, the resistance mechanisms of *Candida* biofilms to antifungal therapy are not fully understood. It is accepted that the antifungal resistance of biofilms is a complex multi-factorial phenomenon that includes alterations or overexpression of target molecules, active extrusion of antifungal agents through efflux pumps and their limited diffusion in the matrix, *stress* tolerance, cell density and presence of persister cells [89]. Formation of mature biofilms and subsequent production of extracellular matrix is strongly dependent on species, strains and environmental conditions [90,91]. Concerning the vaginal environment, *Candida* species can form biofilms on vaginal epithelium [92] and have high capacity to produce biofilms on IUDs promoting VVC [93,94]. *Candida* biofilms have been studied primarily on abiotic surfaces because almost all device-related infections involve growth in the form of biofilm [95]. Recently, biofilm formation on biotic surfaces has received some attention, and Harriott et al. [92] showed, for the first time, that *C. albicans*

forms biofilms *in vivo* on the vaginal mucosa. These investigators also reported that *Candida* biofilm formation *in vivo* requires regulators of biofilm formation (*BCR1*) and morphogenesis (*EFG1*), which were previously identified as necessary genes for biofilm formation on abiotic surfaces [96,97].

The ability of *Candida* species to form biofilms on the vaginal mucosa is an important clinical issue since the recalcitrance of these biofilms to the conventional antifungal therapy may prevent complete eradication of the microorganisms from the vaginal lumen and might explain the frequent recurrence of VVC. In addition, it has been realized that many *Candida* infections are directly linked to biofilms in which multiple species coexist, making the therapeutic management of these infections extremely difficult. Most studies are on mono-species biofilms of *Candida*, and information on mixed-species *Candida* biofilms or *Candida*-bacteria combinations is still scarce [98]. Mixed-species biofilms can be difficult to both diagnose and treat, requiring complex multidrug treatment strategies. Mixed biofilms, especially *Candida*-bacteria biofilms, can cause a dilemma for clinicians because antimicrobials directed towards one species often facilitate non-targeted organisms to continue the infection [98]. Studies report that both vaginal *Candida* species [92] and bacterial pathogens, as *Gardenerella vaginalis* [99], which cause bacterial vaginosis (BV), have the ability to form biofilms on the vaginal mucosa. However, the formation of mixed *Candida*-bacteria biofilms on the vaginal mucosa is still understudied. Some studies have shown that approximately 20-34% of RVVC samples contain vaginal bacterial pathogens such as *Streptococcus agalactiae* and *G. vaginalis* [100,101], possibly due to mixed biofilms with *Candida* species and these bacteria. Concerning mixed biofilm formation on IUDs, a survey on biofilms formed on those devices confirmed the presence of *C. albicans* as well as multiple bacterial pathogens such as *S. agalactiae*, *Escherichia coli* and *Bacteroides* species [102].

Although vaginal infections are an extremely common reason for women to seek care, there is little known about the prevalence of mixed infections, particularly VVC and BV. Recently, Rivers et al. [103] published the first article to present the prevalence of BV, VVC, yeast colonization, and mixed infection. In this study BV was diagnosed in 72.5% of the participants and VVC in 15.7%. Among women with BV, 33.1% were colonized with yeast and the overall prevalence of BV/VVC mixed infection was 4.4%. The authors suggested that the presence of infection/colonization with yeasts likely predisposes women to VVC after treatment of BV with antibiotics. In fact, some studies reveal that VVC is a common side effect of BV treatment with metronidazole or clindamycin [104,105]. Thus, women with BV that also have yeast residing in the vaginal ecosystem can either exhibit failure of symptom resolution from therapy targeted at one infection or development of VVC from exposure to antibiotics. The little information available on the prevalence of vaginal mixed infections is likely due to the fact that most

vaginal infections are diagnosed empirically without the aid of objective data. The lack of awareness to the extent of this problem likely leads to under recognition of mixed infections, resulting in inadequate therapy.

1.4.3 Extracellular hydrolytic enzyme production

Candida species secrete several hydrolytic enzymes, which play an important role in adhesion, tissue penetration, invasion and destruction of host tissues [106]. The enzymes most frequently implicated in *Candida* pathogenicity are secreted aspartyl proteinases (Saps), but phospholipases, lipases and haemolysins are also involved in *Candida* virulence [73].

Saps facilitate adhesion to host tissues and their damage, and are related with changes in the host immune response [107]. To date, ten *SAP* genes (*SAP1-10*) were identified in *C. albicans* [108], three (*SAPP1-3*) in *C. parapsilosis* [109] and at least four (*SAPT1-4*) in *C tropicalis* [110,111], but in these NCAC species most of the genes remains uncharacterized. In the case of *C. glabrata*, a family of 11 cell surface-associated aspartyl proteases (CgYps1-11), also referred as yapsins, were identified, and some of them were demonstrated to have a role in the regulation of pH homeostasis under acid conditions [112,113]. In fact, in contrast with other types of proteinases, Saps show proteinase activity only under acidic conditions (pH ≤ 4.0) [114]. This feature is important for VVC, because the vaginal environment is acidic (pH around 4) [11], providing conditions suitable for activity of Saps. Mohandas and Ballal [115] detected higher proteinase activity in vaginal isolates than in urinary and respiratory isolates, of candidiasis-infected patients, relating Sap production to the site of strain isolation. Furthermore, several studies have reported higher expression of some *SAPs* and higher proteinase activity by *Candida* species isolated from women with VVC than from asymptomatic vaginal *Candida* carriers [116,117]. It has been also demonstrated that the expression of *C. albicans SAP1*, *SAP2* and *SAP3* has a strong and specific correlation with VVC [116,118,119]. Additionally, Monroy-Pérez et al. [120] reported that all *C. albicans SAP* genes are expressed in a model of RHVE, suggesting that aspartyl proteases play an important role in the pathogenesis of the infection. Importantly, it has been reported that some Saps, particularly Sap2, are proinflammatory proteins *in vivo* and can mediate the acute inflammatory response of vaginal epithelial cells to *C. albicans*, being suggested that vaccine-induced anti-Sap antibodies could contribute to control VVC [19].

Phospholipases hydrolyse one or more ester bonds in glycerophospholipids contributing to host cell membrane damage and to the adhesion of yeasts to host tissues. Several *Candida* species have the

ability to produce extracellular phospholipases, but this ability is highly strain dependent and NCAC species produce significantly lower levels compared with *C. albicans* [121]. Mohandas and Ballal [115] found a greater number of phospholipase producing strains in vaginal isolates of patients with candidiasis than in respiratory and skin isolates. In *C. albicans*, seven phospholipase genes have been reported (*PLA*, *PLB1-2*, *PLC1-3* and *PLD1*) [122]. Naglik et al. [119] detected expression of *PLB1* and *PLB2* in vaginal washes of VVC infected women and of asymptomatic vaginal *Candida* carriers, reporting lower levels in healthy women. Furthermore, Alves et al. [49] detected expression of *PLB* and *PLD* gene families in RHVEs infected with several *C. albicans* strains. The highest expression level was exhibited by *PLD1* indicating a potential role of this factor in RHVE damage.

Lipases are involved in the hydrolysis of triacylglycerols and their activity has been associated to *Candida* adhesion and damage of host tissues and affects immune cells [123]. In *C. albicans*, lipases are coded by 10 genes (*LIP1-10*), similar sequences were identified in *C. tropicalis* and two lipase genes were detected in *C. parapsilosis* (*CpLIP1-2*), but none in *C. glabrata* [73]. These enzymes are less studied than Saps and phospholipases, especially concerning their specific association with the anatomical site of infection. Haemolysins produced by *Candida* species degrade haemoglobin, facilitating recovery of iron, being essential to survival and persistence in the host. The production of these proteins was already described in several *Candida* species including *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, however the genetic expression of haemolytic activity is poorly understood [124].

1.4.4 Filamentous forms development

A reversible morphological alternation between unicellular yeast cells and filamentous phase (hyphae and pseudohyphae) is an important virulence factor of some *Candida* species [73]. *Candida albicans* and *C. dubliniensis* have the ability to form hyphae and/or pseudohyphae, *C. parapsilosis* can generate pesudohyphae, *C. tropicalis* pesudohyphae and possibly true hyphae and *C. glabrata* grows only as blastoconidia [73]. Filamentous forms give more mechanical strength, enhancing colonization and invasion of host tissues and demonstrate increased resistance to phagocytosis [73,125]. The yeast-hyphal switch is affected by environmental factors and is related with other virulence factors. For instance, phospholipase D (*PLD1*) is necessary for yeast-to-hyphal transition in *C. albicans* [126] and the expression of some Saps genes (*SAP4-6*) occurs specifically during hyphal development [127]. Taylor et al. [128] detected association of *SAP4* and *SAP5* expression with hyphal formation during

vaginal candidiasis in mice. Furthermore, Alves et al. [49] in a study with RHVE infected with *C. albicans* detected expression of the Hyphal Wall Protein (*HWP1*) gene, which is a specific hyphae-adhesin responsible for a covalent attachment of the yeast to host epithelial cells. Interestingly, in RHVE co-infected with *C. albicans* and *C. glabrata*, being the latter a non-hyphae producer, the investigators detected an up-regulation of *HWP1* that may have contributed for the enhanced tissue invasion by *C. glabrata* observed in the presence of *C. albicans*.

1.4.5 Phenotypic switching

The colonies of most *Candida* species can switch among different phenotypes at high frequency. In *C. albicans* white-phase round-ovoid cells can switch to opaque-phase cells which are elongated or bean-shaped [129]. It has been identified that the genes *MTLa1-2*, *WOR1-2*, *CZF1* and *EFG1* regulate the white to opaque switching [130,131]. Furthermore *SAP3* and *EFG1* are expressed specifically in white cells whereas *SAP1-2* are expressed specifically in opaque cells [132]. Comparatively to *C. albicans* the phenotypic switching of NCAC species is less understood. It has been reported that *C. glabrata* can undergo "core switching" that results in white, light brown, dark brown, very dark brown or irregular wrinkle colonies. Brockert et al. [133] showed a predominance of dark brown colonies among vaginal isolates of patients with VVC. The phenotypic switching alters several virulence traits including hyphae formation, drug resistance, adhesion and Saps secretion, potentially affecting survival in specific anatomical-sites and promoting infection [134,135]. The precise contribution of phenotypic switching to VVC is still not clear but it has been demonstrated that vaginal isolates obtained during VVC and RVVC are in high-frequency mode of switching [136,137]. Furthermore, in each recurrence of one patient with RVVC, different colony phenotypes were observed but DNA genotyping remained identical [137].

All virulence factors that mediate *Candida* pathogenicity are influenced by the specific environmental conditions of each anatomical site. Thus, the disturbance of the normal environment of the lower female genital tract may increase the risk of infection by *Candida* species. Next will be reviewed the knowledge about risks factors predisposing for VVC.

1.5 Risk factors for VVC

The vaginal flora is highly dynamic with a local microbial system. There is a balance between *Candida* vaginal colonization and the host environment that can be disturbed by physiological or non-physiological changes, making the colonization site favourable for the development of yeasts. Healthy women can develop VVC sporadically however this infection is often attributed to the presence of host-related and behavioural factors that disturb the vaginal environment, promoting VVC. Proposed host-related risk factors include pregnancy, hormone replacement, uncontrolled diabetes mellitus, immunosuppression, antibiotics and glucocorticoids use and genetic predispositions [20] (Figure 1.2). Behavioural risks factors for VVC include use of oral contraceptives, intrauterine device, spermicides and/or condoms, and also some sexual, hygienic and clothing habits [20,138] (Figure 1.2).

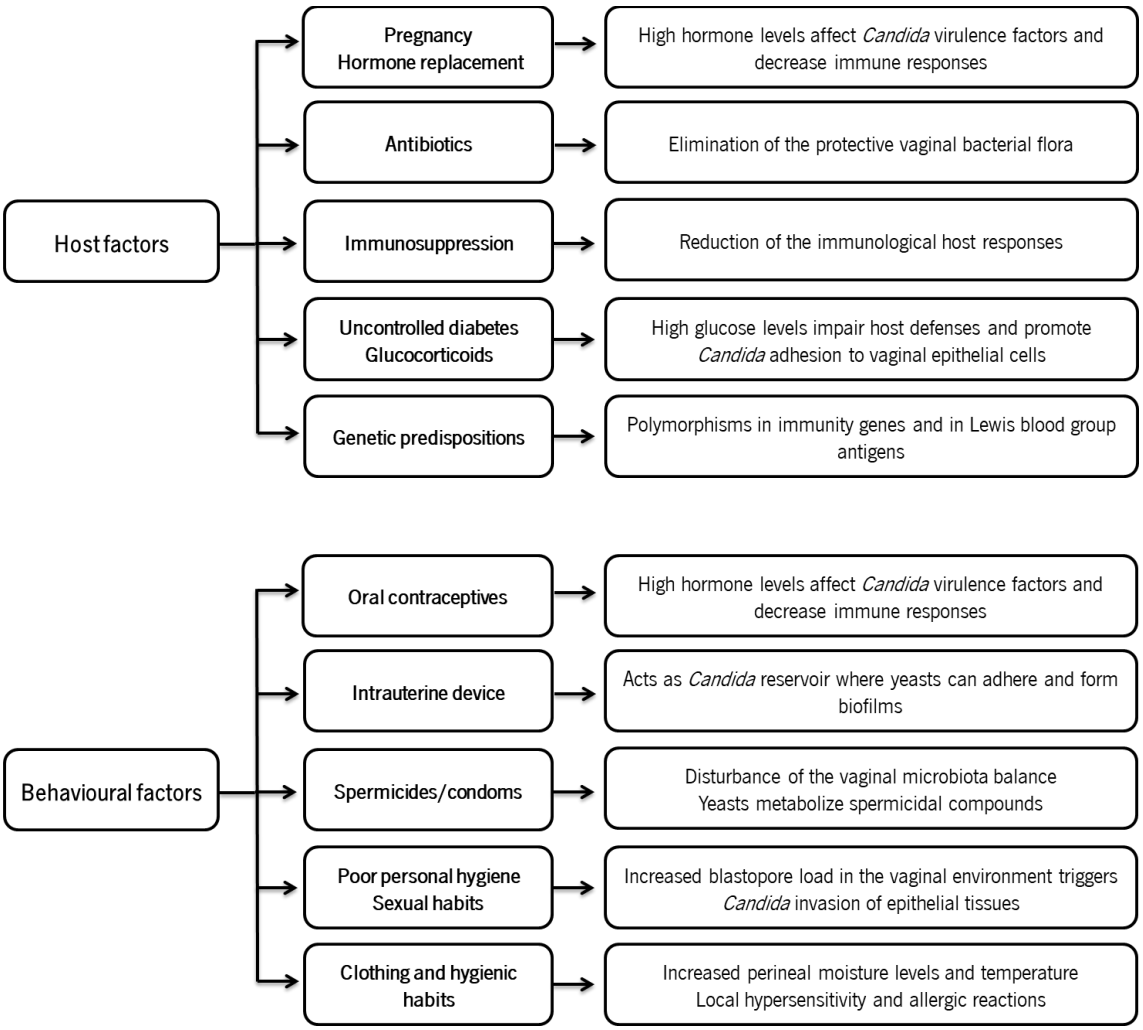


Figure 1.2 Host-related and behavioural risk factors for vulvovaginal candidiasis and respective effects.

1.5.1 Host factors

1.5.1.1 Pregnancy and hormone replacement therapy

Pregnancy has been considered an important risk factor for the development of VVC because several studies report high incidence of the disease in pregnant women. Table 1.3 shows studies published during the last years, concerning the incidence of VVC in pregnant and non-pregnant women.

Table 1.3 Incidence of VVC in pregnant and non-pregnant women.

Country	Pregnant women (% with VVC)	Non-pregnant women (% with VVC)	Reference
Brazil	16.4	8.6	[52]
India	66.7	20.3	[32]
India	76.0	31.0	[35]
India	10.0	7.7	[36]
India	28.2	7.9	[139]
Greece	29.1	7.9	[44]
Belgium	32.0	19.3	[140]
Tunisia	44.1	29.9	[27]
Nigeria	47.7	20.3	[141]
Nigeria	40.0	not included	[23]
Nigeria	91.0	not included	[142]
Kenya	90.4	not included	[143]
Burkina Faso	58.0	not included	[144]
Nepal	75.3	not included	[145]

The epidemiologic studies have been consensual in reporting higher prevalence of the disease in pregnant women than in non-pregnant patients, although the incidence varies depending on the locations and population studied. In the last years, many studies of VVC incidence in pregnant women were performed in India and Nigeria (Table 1.3), probably because these countries have high birth rate and thus, have interest in studying pregnant-related diseases. In fact, the highest incidences of VVC in pregnancy comparatively to non-pregnancy were reported by some Indian [32,35] (66.7–76% vs 20.3–31.0%) and Nigerian studies [141] (47.7 vs 20.3%). Additionally, some studies in Nigeria, as well as in Kenya, Burkina Faso and Nepal, only included the incidence of VVC in pregnant women without a non-

pregnant control, but all of them reported high incidence of the disease in pregnancy (40.0 to 91%) [23,142–145] (Table 1.3). In addition, since the vaginal colonization is a prerequisite for symptomatic VVC, some studies have also studied the incidence of *Candida* colonization in non-symptomatic pregnant women. These studies reported higher prevalence of vaginal colonization by *Candida* in pregnant women than in those who were not pregnant, indicating that pregnancy increases vaginal colonization [146,147].

The high incidence of VVC in pregnancy has been attributed to the increase of sex hormones secretion in pregnancy. In fact, during pregnancy VVC prevalence is higher in the last trimester, when levels of hormones are more elevated, even though symptomatic recurrences are common throughout pregnancy [140,143,148]. Furthermore, in non-pregnant women the infection is more incident during the luteal phase of the menstrual cycle, which is the phase with the highest hormone secretion [149]. Kalo and Segal [150] also demonstrated that the level of *in vitro* adherence of *C. albicans* to human exfoliated vaginal cells has a correlation with the hormonal status of the cell donors, being higher in cells of pregnant women and of those in luteal phase of the menstrual cycle. The hormonal dependence of VVC is also evidenced by the fact that the disease is uncommon in pre-puberty and post-menopause, except in women taking hormone replacement therapy (HRT) [42,140]. HRT is used to counter adverse the consequences associated with the decrease in hormones secretion in post-menopause, such as osteoporosis, diabetes mellitus, cardiovascular disease and neurodegeneration [151,152], however it has been considered a risk factor for the development of VVC. Higher incidence of VVC has been reported in women receiving HRT (26.0 to 29.4%) than in post-menopausal women without HRT (4 to 12.6%) [140,153,154]. All authors agree that the high hormonal levels of pregnant women and of those receiving HRT are primarily responsible for the relation of those conditions with VVC. However, emotional stress, suppression of immune system and eating habits of sugar rich containing food may also contribute to the development of VVC in those women [9].

The two types of female sex hormones are estrogens and progestins. The most important progestin is progesterone, which is secreted by the *corpus luteum*, placenta and adrenal cortex. During the luteal phase of the menstrual cycle significant amounts of progesterone are secreted and during pregnancy its levels increase about 10 times. Progesterone is important in many vital actions of the woman, such as endometrial and breast development, maintenance of pregnancy, decrease of insulin action and increase of sodium excretion by the kidneys [155]. The main estrogen in pre-menopause is β-estradiol and in post-menopause is estrone, which has a lower estrogenic potency than β-estradiol. Significant amounts of estrogens are secreted by the ovaries and during pregnancy high amounts of

these hormones are secreted by the placenta increasing about 30 times their levels. Estrogens perform many essential actions in women, including the development of secondary sex characteristics, uterine growth and conservation of the vaginal mucosa [155]. Despite being accepted that VVC has a hormonal dependency, the mechanisms by which progesterone and estrogens act in the disease are not fully understood. One proposed mechanism is associated with the increase of glycogen load in vaginal epithelium when the levels of progesterone and estrogens increase. The walls of vagina are lined with the pavement of epithelial cells, which produce glycogen in proportion to hormonal levels and thus the state of the vaginal mucosa reflects female hormonal status in different lifetime stages [153]. The production of glycogen by hormone stimulated epithelium possibly contributes to the proliferation of *Candida* species when host hormones exceed a certain level, because glycogen provides an excellent nutritional source of carbon for *Candida* growth [100,153]. In addition to the effect on vaginal epithelium it has been also proposed that sex hormones inhibit aspects of both innate and adaptive immunity at systemic or local level. In fact, *in vitro* studies on vaginal epithelial cells showed that progesterone and estrogens inhibit *Candida*-specific human peripheral blood lymphocyte (PBL) responses [156] and that estradiol significantly reduces antimicrobials production (HBD2 and elafin) [157]. Furthermore, analyses of cervical-vaginal secretions demonstrate that chemokines and cytokines (IL-6 and IL-8), antimicrobials (HBD2 and lactoferrin) and levels of IgA and IgG antibodies are depressed by 10- to 100-fold at mid-cycle, remain depressed for 7-10 days and rise to proliferative levels in the end of the menstrual cycle [158]. In addition, Nohmi et al. [159] reported that physiological blood level of progesterone on pregnant women clearly suppresses mice neutrophil anti-*Candida* activity.

Besides the effects on the host it has been also shown that progesterone and estrogens have direct effects on *Candida* cells, possibly contributing to VVC. One direct effect of the hormones is the stimulation of estrogen and progesterone cytosolic receptors, which have been already identified in several *Candida* species. An estrogen-binding protein (EBP), that displays high affinity for estradiol and estrone, was identified and characterized in *C. albicans* [160] and an estrogen binding system was also detected in *C. glabrata* [161]. In addition, a corticosteroid-binding protein (CBP) that exhibits high affinity for corticosterone and progesterone, but low affinity for estrogens, was identified in *C. albicans*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* [162,163]. However, the effect of hormones on *Candida* virulence is not entirely clear. The studies have shown that progesterone [159,164,165] and estradiol [166–168] may stimulate, inhibit or not affect *C. albicans* growth and/or filamentous forms formation, depending on strain and experimental conditions. Interestingly, Alves et al. [164] showed that the presence of progesterone reduces the ability of *C. albicans* to develop biofilms

and filamentous forms at pH 4 (Figure 1.3) and decreases the expression levels of the biofilm related genes *BCR1* and *HWP1*. These investigators also showed that *C. albicans* colonization and invasion of RHVE decreased substantially in the presence of a high level of progesterone. Importantly, progesterone and estradiol were found to increases *C. albicans* expression of *CDR1* and *CDR2*, which encode multidrug transporters of the ABC family, suggesting an involvement in drug resistance [165,167]. Additionally, both hormones were found to increase *C. albicans* stress response, especially to heat and oxidative stresses [165,169].

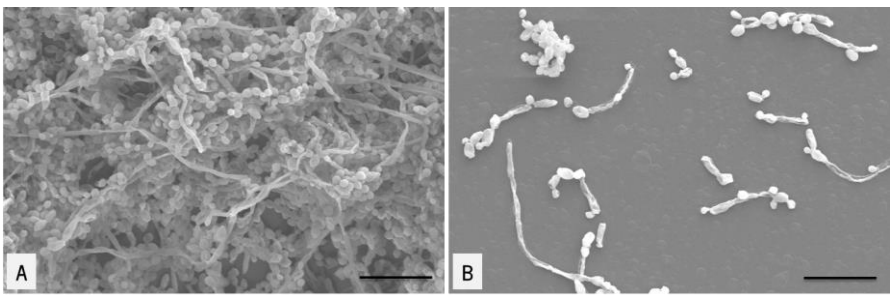


Figure 1.3 Scanning electron microscopy images of *Candida albicans* ATCC biofilms formed in the **(A)** absence and **(B)** presence of 2 μM of progesterone. Original magnification was x1000 and the scale bars correspond to 20 μm [164].

Since VVC incidence is higher and cure rates are lower in conditions with high hormone levels such as pregnancy and HRT, the therapy must be appropriate for these patients [170]. VVC in pregnancy does not usually harm the unborn child but if the disease persists the baby can get infected (oral thrush) during delivery, which can be a very serious health problem in premature babies. Furthermore, infants with oral thrush can give rise to nipple candidiasis in breast-feeding mothers [171]. In addition, some authors report reduction of preterm birth in pregnant women treated for VVC with clotrimazole [172,173]. It is has been also shown that a large proportion of women with RVVC first appear with the infection during pregnancy [174]. Thus, in order to avert the complications associated with VVC in risk groups such as pregnant women and those with HRT early diagnosis and prompt treatment are very important.

1.5.1.2 Antibiotics

The use of antibiotics (vaginal or systemic) has been considered a risk factor for the development of VVC. Although some studies failed to show association between antibiotic treatment and occurrence of VVC [175,176], many others found higher VVC prevalence in women that have taken antibiotics than in those who have not been treated with antibiotic agents (Table 1.4). Table 1.4 highlights the most relevant reports that studied the prevalence of VVC in women treated and not treated with antibiotics and/or incidence of antibiotic use in women with and without VVC.

Table 1.4 Incidence of VVC in women who used or not antibiotics and incidence of antibiotic use in women with and without VVC (ni - not included in the study; ns - not shown in the study).

Country	Incidence of VVC (%)		Incidence of antibiotic use (%)		Reference
	Antibiotic users	Not antibiotic users	With VVC	Without VVC	
India	57.5	11.8	ni	ni	[35]
Greece	18.0	8.2	ni	ni	[44]
Italy	55.2	41.0	19.3	11.9	[177]
Italy	45.5	39.2	18.3	11.4	[178]
Turkey	ni	ni	29.5	6.8	[179]
Brazil	ni	ni	8.2	0.7	[59]
USA	ni	ni	13.0	6.0	[149]
Nigeria	ni	ni	16.0	ns	[23]
Israel	ni	ni	23.0	ns	[46]

Studies in India [35], Greece [44] and Italy [177,178] showed an increased incidence of VVC in women that have taken antibiotics than in those who have not taken them (18.0 – 57.5% vs 8.2 – 41.0%). Studies in Italy [177,178], Turkey [179], Brazil [59], USA [149], Nigeria [23] and Israel [46] showed an increased incidence of antibiotic use in women with VVC than without VVC (8.2 – 29.5% vs 0.7-11.9%) (Table 1.4). Thus, all these studies propose that antibiotic treatment is a risk factor for VVC. These epidemiologic data may have been influenced not only by the location where the study was made but also by type of antibiotic agents used and the duration of antibiotic treatment. In fact, the duration of antibiotic use is directly related to an increase of VVC prevalence [177] and broad-spectrum antibiotics (e.g. tetracycline, ampicillin and cephalosporin) are more likely to favour yeast infections

compared to narrow-spectrum antibiotics (e.g. erythromycin, lincomycin) [180,181]. Furthermore, it has been shown that antibiotic agents also predispose to RVVC [177–179], in some cases with more significance than for sporadic acute VVC [179]. In addition, some studies found an increased incidence of asymptomatic vaginal colonization by *Candida* after an antibiotic treatment than before it (37% vs 18 – 21%), and a subsequent development of VVC after the antibiotic intake in 20 – 22% of initially colonized women [182,183]. These studies also report that only women already colonized with *Candida* are at risk of VVC following antibiotic treatment.

It is thought that the association of VVC and antibiotics is due to the fact that antibiotic use leads to the depletion of the vaginal bacterial microflora, which represents the dominant vaginal defence mechanism against *Candida* [184]. The vaginal microbiota of healthy premenopausal woman is predominantly populated by *Lactobacillus* species, the most common of which are *L. iners*, *L. crispatus*, *L. gasseri*, *L. jenesenii,* followed by *L. acidophilus, L. casei, L. vaginalis, L. Salivarius* [185]. Lactobacilli are thought to be involved in several defence mechanisms against *Candida* microorganisms. One proposed mechanism is that *Lactobacillus* species compete with *Candida* species for nutrients, however, a "shoulder-to-shoulder" survival for lactobacilli and *Candida* has been shown on an experimental basis, proving that this is not the most effective mechanism [6]. More importantly, lactobacilli compete with *Candida* cells for adhesion sites, such as epithelial receptors, to which *Lactobacillus* has higher affinity [186,187]. Some studies have found a decreased adhesion of *C. albicans* to vaginal epithelial cells when *Lactobacillus* is present in comparison with the adhesion observed when only *Candida* is present [187,188]. Furthermore, lactobacilli secrete biosurfactants that physically decrease *Candida* binding. Velraeds et al. [189] found a 50% decrease in the adhesion of *C. albicans* to a silicone rubber filled with a biosurfactant (surlactin) compared with the adhesion levels on silicone rubber without surlactin. Lactobacilli also produce bacteriocins and bacteriocin-like substances that inhibit *Candida* growth. For instance, Okkers et al. [190] showed that a bacteriocin-like peptide (pentocin TV35b) isolated from *Lactobacillus pentosus* inhibits *C. albicans* growth. Moreover, most *Lactobacillus* strains release hydrogen peroxide (H_2O_2) and fatty acids that inhibit *Candida* overgrowth and invasive hypal formation [186]. Some studies suggested that pregnant women whose vaginas were colonized by H_2O_2-producing lactobacilli were less likely to have symptomatic VVC than those colonized with H_2O_2-nonproducing vaginal lactobacilli [191,192]. Furthermore, Boris and Barbés [186] detected lower presence of H_2O_2-producing population in women with VVC than in healthy women. In addition, Xu and Sobel [193] showed that *L. delbrueckii* strains, which produce the largest amounts of H_2O_2, have

the strongest and fastest growth inhibition effect against *C. albicans*, amongst several *Lactobacillus* strains isolated from vaginas of healthy women.

The vaginal microbiota is also rich in lactic acid-producing *Lactobacillus*, which provide adequate acidity to the vaginal environment (pH around 3.6-4.5), hindering the proliferation of most pathogens [194]. However, *Candida* species are exception because they proliferate in acidic environment. Patients with VVC have normal vaginal pH, while patients with other vaginal infections such as bacterial vaginosis, trichomoniasis and atrophic vaginitis have higher vaginal pH (>4.5) [35,139]. Moreover, it has been detected higher vaginal pH in women with *C. glabrata* infection than in those with VVC due to *C. albicans* [35,195]. In fact, a more alkaline pH such occurs with concomitant bacterial vaginosis has been postulated as a risk factor for *C. glabrata* vaginal infections [15]. In addition, increased vaginal pH and predominance of NCAC species, especially *C. glabrata*, has been detected in post-menopausal women [56,58]. In these women the vaginal pH is enhanced by the hormonal depletion that leads to a reduction of glycogen production in vagina, which is essential to the vaginal bacteria anaerobic metabolism of glycogen to acid lactic [196].

It is known that the pH of the host niche is a significant environmental signal that determines the biological response and survival of pathogens [197]. Thus, the fact that *Candida* organisms can adapt to environments with different pH, makes them important human pathogens. In *C. albicans*, pH-regulated genes such as *PHR1* and *PHR2* have been identified. *PHR1* is only expressed at pH levels above 5.5 and its functional homolog *PHR2* is highly expressed when pH levels are below 5 and is not expressed with pH above 6 [198]. Studies *in vitro* found that both genes encode a function required for *C. albicans* morphogenesis. Deletion of *PHR1* result in inability to form normal yeast or hyphal morphology at an alkaline pH, but not at an acidic pH, and these mutants lost the ability to cause systemic disease but are fully capable of causing vaginal infection. Conversely, deletion of *PHR2* result in a morphogenic defect at an acidic pH and these mutants lost the ability to cause vaginal infection but are virulent in systemic infections. Besides the morphogenic defect, both mutants also exhibit altered growth rates at the restrictive pH [197,198]. In addition, our research group studied planktonic cultures of *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* grown at pH between 3 and 8, and we found that these species can proliferate at all the pH values tested. We also studied the influence of pH on the capacity of *C. tropicalis* isolates to form biofilms and we found that the pH increase lead to an increased biofilm formation capacity (Figure 1.4) and increased hyphal length, suggesting that *Candida* species are able to adapt to vaginal environmental pH alterations [199].

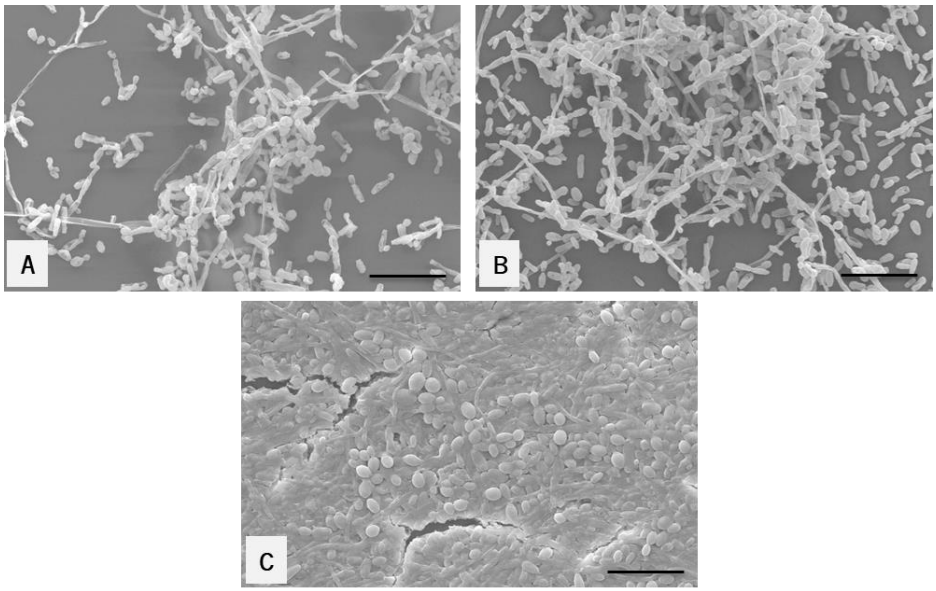


Figure 1.4 Scanning electron microscopy images of *Candida tropicalis* ATCC biofilms formed at **(A)** pH 4, **(B)** pH 7 and **(C)** pH 8. Original magnification was x1000 and the scale bars correspond to 20 μm.

Several studies have shown that vaginal *Lactobacillus* could provide colonization resistance and prevent germination of *Candida* organisms [187,188,200]. Thus, it is thought that the suppression of this protective barrier due to antibiotic use may lead to VVC development. However, some studies found that the absence of vaginal lactobacilli does not increase acquisition of VVC in the absence of antibiotic intake, suggesting that the interaction between antibiotic use and *Candida* infection involves other mechanisms besides bacterial suppression [105,201]. Furthermore, some studies reported that the use of vaginal or oral probiotic *Lactobacillus* does not prevent VVC after antibiotic treatment [202,203]. However, contradicting these results, some studies found that probiotic *Lactobacillus* administration slows or prevents *C. albicans* growth [160,184,204]. In fact, *Lactobacillus*-based probiotics are being pursued as an adjunct to conventional treatment for RVVC [205,206]. Although the mechanisms involved in VVC promotion by antibiotic use are still not entirely clear, it is accepted that it is an important risk factor for the development of VVC.

1.5.1.3 Immunosuppression

All *Candida* infections are dependent on the host and therefore its immune response is crucial in the host-pathogen interaction [207]. In the vaginal environment effective anti-*Candida* defence mechanisms prevent *Candida* infection but allow long-term colonization of *Candida* organisms as

vaginal commensals[20]. Thus, immunosuppressive conditions such as HIV-infection [23,208], cancer chemotherapy, glucocorticoids therapy [209], organ transplant, cancer, diabetes mellitus, tuberculosis [23], and any chronic debilitating illness can increase the chances of developing VVC. Furthermore, it has been proposed that some women with RVVC may have a dysfunction in the normal protective immune response, acquired from early exposure to *Candida* organisms [210,211].

The most studied immunosuppressive condition predisposing to VVC is the HIV infection, probably because VVC may also increase the risk of HIV acquisition [22,212]. It is though that up to 50-70% of HIV-infected patients develops VVC [6]. Some studies have reported increased odds for vaginal *Candida* colonization [213] and symptomatic VVC [208,214] in HIV-infected women than in HIV-negative patients. Moreover, Apalata et al. [214] found a negative association between the use of highly active anti-retroviral therapy and the presence of symptomatic VVC in HIV-infected women. In addition, NCAC species have been found more frequently in HIV-infected women than in non-infected patients [53,63]. Nevertheless, no studies indicate that HIV-infected women with VVC are less likely to respond to the antifungal therapy or have a more severe fungal infection than HIV-negative women [208,215].

Protection against potential pathogens in the female reproductive tract is provided by the innate and adaptive immunity systems. Innate immune system is recognized as the first line of defence, preventing and controlling invasion of pathogens [216]. Cells of host's innate immunity express receptors that recognize pathogen associated molecular patterns (PAMPs) found in groups of microorganisms [217]. Some of these receptors recognize molecular patterns common to all fungi, while others bind to specific *Candida* components [218,219]. The main classes of pattern recognition receptors that recognize *Candida* are Toll-like receptors (TLR) and lectin-like receptors (LR) families [220]. In addition to cell-membrane receptors, the circulating mannose binding lectin (MBL) recognizes and binds strongly to *Candida* surface mannans, enhancing complement activation and thus inhibiting *Candid*a growth [221,222]. Genetic polymorphisms in recognition receptors such as dectin-1 [223] and MBL [224,225] have been associated with increased susceptibility to RVVC.

The cells involved of the innate immune system that express cell-membrane receptors include neutrophils, dendritic cells (DC), macrophages and vaginal epithelial cells (VEC) [216]. The depletion of neutrophils was reported to increase the susceptibility to VVC in mice, however their role on anti-*Candida* protection at the vaginal level is controversial [226,227]. DC are scattered throughout different portions of the vagina and cervical epithelia, bind to *Candida* organisms and transmit differentiation and activation signals leading to inflammation, cell activation and induction of adaptive immunity [228]. Regarding macrophages, their stimulation through *Candida* recognition has been shown to induce the

synthesis of nitric oxide (NO) and oxygen radicals, compounds toxic for *Candida* organisms. Rosentul et al. [229] detected lower NO concentrations in vaginal secretions of patients with RVVC than of healthy patients. Furthermore, macrophages and DC contain cell surface receptors that recognize and bind MBL, promoting opsonization of MBL-bounded microorganisms [230]. In addition, VEC have been suggested to play a crucial role in the defence mechanisms against VVC [231,232]. Vaginal surface area ranges from 65 to 108 cm^2 [233] and is lined by multiple layers of squamous non-keratinized epithelial cells [234], which are the primary cells that *Candida* interact with, at the vaginal mucosa. VEC not only provide a physical barrier against pathogen entry in the lower reproductive tract but also recognize and process antigens. Furthermore, VEC secrete immune mediators such as chemokines and cytokines, which recruit and activate immune cells of both the innate and adaptive immune systems [216,235]. In addition, they also secrete molecules with potent antimicrobial activity such as human α-defensin-5 (HD5), β-defensins 1-4 (HBD1-4), elafin and SLPI (secretory leukocyte protease inhibitor) [236,237]. These antimicrobial peptides have been shown to be effective at inhibiting several pathogens including *Candida* organisms [238,239], however a definite anti-*Candida* role for any specific peptide in the vaginal environment has not been established. It has been shown that VEC from women with RVVC exhibit reduced *in vitro* anti-*Candida* activity comparatively to cells from healthy women [231,240]. Although this yet to be demonstrated *in vivo*, these results suggest that VEC provide an innate host resistance mechanism against *Candida* and their reduced anti-*Candida* activity may contribute to RVVC.

Candida recognition by the innate immunity triggers a sequence of events, including cytokine and chemokine production, that lead to the activation of adaptive immunity. While the innate immune system is activated immediately after pathogen recognition, it takes several days for the adaptive system to become functional. The adaptive immunity encompasses fungal-specific defence mechanisms including destruction of specific pathogens directly or indirectly by T cells (cell-mediated immunity) and protection through specific antibodies produced by B cells (humoral immunity) [216]. Depressed T-cell mediated immunity has been suggested to be associated with increased susceptibility to VVC and RVVC in women with HIV-infection, transplanted organ, under glucocorticoids therapy and cancer chemotherapy [23,209,214,241]. It has been suggested that most vaginal T-cells migrate to the vaginal epithelium in response to local antigenic stimulation and/or inflammatory chemokines [242,243]. In fact, the vaginal mucosa is suggested to be an immunocompetent tissue capable of a compartmentalized cell-mediated response independent of systemic cell-mediated immunity [210,244]. Cell-mediated immunity through CD4 and CD8 T-cells has been widely demonstrated to be essential for *Candida* protection at the vaginal level [245,246]. Additionally, some studies have shown that Th1 T-

cells produce pro-inflammatory cytokines, such as interferon-γ, which may contribute to VVC protection [247,248]. However, the protective role of Th1 cells against VVC is still controversial [226,249]. In contrast to Th1 cells, Th2 cells produce anti-inflammatory cytokines as IL-4, which inhibits macrophage-mediated anti-*Candida* responses and protective pro-inflammatory Th1-derived cytokines [250]. Rosentul et al. [229] showed that a genetic propensity to an overwhelming release of anti-inflammatory Th2-derived cytokines contributes to RVVC development. In addition, Pietrella et al. [251] showed that Th17 cells (which belong to a different lineage from that of Th1 and Th2) and their IL-17 production have an important role in the immune response to VVC.

Similar to T cells, B cells and immunoglobulin (Ig)-secreting plasma cells, migrate to the vaginal epithelium, where they are normally absent, in response to an antigenic stimulation [210,243]. The local production of antibodies by these cells might be a quick mechanism for combating pathogenic microorganisms without the need to wait for the beginning of the systemic immune response. The exact mechanism by which antibodies may protect against *Candida* infections is unknown, but appears to include inhibition of adhesion, opsonization, neutralization of enzymes related to virulence and direct fungicidal activity. Immunoglobulin A (IgA) and IgG are the predominant Ig classes found in vaginal secretions, suggesting that they represent the dominant Igs in the female lower genital tract [252,253]. Carvalho et al. [252] found a marked response of IgA, IgG1 and IgG4 in vaginal washes of symptomatic women with VVC comparatively to symptomatic women without *Candida*, suggesting that the synthesis of those antibodies was stimulated by the presence of the fungus. In fact, several studies have shown that vaginal colonization or infection by *Candida* induces anti-*Candida* antibodies production [210,252,254] but whether these humoral responses are important to avoid VVC is still controversial. Some studies have reported normal or high quantity of *Candida*-specific antibodies in vaginal secretions of women with VVC and RVVC, suggesting little to no protective role for antibodies [244,255]. In contrast, other studies found low levels of anti-*Candida* antibodies in vaginal washes of women with VVC than of healthy colonized patients, proposing that the decrease of antibody levels promoted *Candida* infection [254,256]. Importantly, the secretor component of IgA was demonstrated to attach to *C. albicans* in a non-immune way, blocking *Candida* binding to cellular receptors [254]. This immunoglobulin forms complexes with *Candida* organisms, contributing to the inhibition of *Candida* adhesion to the vaginal epithelium [254,257]. Nevertheless, vaginal secretory immunity can also be the cause of higher susceptibility to VVC. It has been proposed that some women could have IgE-mediated hypersensitivity contributing to the etiology and/or severity of the infection. High levels of IgE have been

found in vaginal secretions of women with VVC and particularly with RVVC, compared to healthy women, suggesting an allergic sensitization to *Candida* antigens [258,259].

1.5.1.4 Uncontrolled diabetes and glucocorticoids

Diabetes mellitus (DM) has been proposed as a risk factor for the development of VVC since higher prevalence of VVC has been found in diabetic women than in non-diabetics. The incidence of VVC in diabetic women ranges between 32 and 67.5% and in non-diabetic patients ranges between 11 and 23% [44,67,68,260]. Furthermore, higher vaginal colonization by *Candida* and higher development of RVVC has been detected in diabetic patients than in non-diabetics [261,262]. Importantly, Atabek et al. [263] isolated *Candida* species in 39% of children with DM between 8–16 years of age, contrasting with the rare vaginal colonization in healthy prepubertal girls [264].It has been also shown that, a large proportion of VVC in diabetic women is due to NCAC species, especially *C. glabrata*. This species has been isolated in 50 – 61% of diabetic patients with VVC whereas *C. albicans* is only detected in 29 – 36% of these women [68,265,266]. In addition, De Leon et al. [267] found that most diabetic type 1 participants were vaginal colonized with *C. albicans* (56%), while *C. glabrata* was the most common isolate among type 2 participants (54%). High predominance of NCAC species in diabetic women may have important implications for VVC treatment because NCAC species are inherently less susceptible to conventional antifungal therapy. In fact, Goswami et al. [68] reported that 81.3% of diabetic patients with VVC continued to show fungal growth following fluconazole therapy, when *C. glabrata* was present, and only 45.4% of patients had persistent *C. albicans* infection. Furthermore, these investigators also found that *Candida* persistence was higher in diabetic women than in the non-diabetic control group (78.6% and 21.5% for *C. glabrata* and *C. albicans*, respectively). These results showed that diabetic patients have less cure rates with fluconazole than non-diabetics, even with the same causative species. In diabetic patients, higher mycological cure of *C. glabrata* VVC has been achieved when they were treated with boric acid vaginal suppositories instead of oral fluconazole [266].

Diabetes mellitus is a metabolic disturbance caused by either the pancreas not producing enough insulin (type 1 DM) or the cells of the body not responding properly to the insulin produced (type 2 DM). As insulin regulates the uptake of glucose from the blood into cells of the body, diabetic patients have elevated plasma levels of glucose [268]. It has been proposed that hyperglycemia is the major cause of increased susceptibility of diabetic patients to VVC. In fact, the risk of *Candida* vaginal colonization and symptomatic VVC is minimized in diabetic women with well-controlled glucose levels

[67,267]. Furthermore, in non-diabetic patients with RVVC, it was described an association between excess of refined sugars consumption and exacerbation of symptomatic VVC [269]. It has been proposed that elevated levels of glucose predispose to VVC by impairing basic mechanisms of host defence and by promoting *Candida* adhesion to vaginal cells [270,271]. Mikamo et al. [271] found that the adhesion of *C. albicans* cells to human VEC is significantly increased in the presence of high glucose levels. It is though that the excess of glucose increases the nutritive substract of *Candida* microorganisms, promoting their adhesion [272]. Nevertheless, Mikamo et al. [271] found that although *C. albicans* adhesion to VEC is induced by high glucose levels, its adhesion to human vaginal epidermal cells is not. These investigators discover that the glucose-induced adhesion is mediated by a *Candida* receptor, the intercellular adhesion molecule ICAM-1, which expression in VEC increases as the glucose levels increase. Additionally, a *C. albicans* glucose-inducible surface protein that promotes adhesion to VEC was also found [273]. Moreover, this protein is structurally and functionally homologous to a complement receptor on mammalian phagocytes and its increase impairs neutrophil phagocytic recognition. Furthermore, glucose binds covalently and irreversibly to the complement C3 inhibiting the attachment of this protein to *Candida* microorganisms, which is the critical determinant of phagocytic recognition [273]. Consistently, Ferreira et al. [274], in a study with rat models, showed that diabetic neutrophils are less effective than those of healthy controls in the phagocytosis and killing of *C. albicans* cells. These investigators suggested that the impaired activity of neutrophils' myeloperoxidase may play an important role in the dysfunction of neutrophils from hyperglycaemic rats. Furthermore Nash et al. [275] reported lower vaginal neutrophils infiltration in diabatic mice colonized with *C. glabrata*. Most of the mechanisms responsible for neutrophils dysfunctions in hyperglycaemic environments are not clear but they can be corrected or at least substantially improved with control of plasma glucose [261,274].

It is though that, patients with hyperglycemia due to other factors than DM are also at high risk of developing VVC. For instance, the use of glucocorticoids has been proposed as a risk factor for VVC [20]. Glucocorticoids are used to treat several human illnesses, such as, autoimmune diseases and cancer and to prevent rejection following organ transplant. However, one of their many undesirable side effects is hyperglycemia caused by increased production of glucose from amino-acids breakdown and resistance to the insulin action [276]. Thus, it can be suggested that the high blood glucose levels that contribute for VVC in diabetic patients, have similar action on glucocorticoids users, leading to an increased risk of VVC development. Furthermore, it was shown that several *Candida* species possess a corticosteroid-binding protein and synthesize a fungal ligand that competes with corticosteroids for

mammalian glucocorticoid receptors, leading to the speculation that exogenous glucocorticoids may directly mediate *Candida* virulence [273]. In addition, the use of glucocorticoids suppresses several immune reactions (which is one of the main reasons for their use), increasing the vulnerability to fungal infections [277]. Although the incidence of VVC in patients with glucocorticoids therapy is unknown, it has been shown that their use increases the incidence of other *Candida* infections, for instance oral candidiasis [278].

1.5.1.5 Genetic predispositions

Since many women develop VVC without any known predisposing factor, some groups of researchers have turned to genetic studies to reveal a possible association of VVC with genetic predispositions [279]. American studies have found that African-American women have increased prevalence of VVC [176] and are more likely to be vaginal colonized by *Candida* [146] compared to Hispanic and white women. Furthermore, black women have decreased vaginal H_2O_2-producing *Lactobacillus* population, which is a protective barrier against *Candida* microorganisms [280]. Thus, a genetic-related predisposition has been proposed as a risk factor for VVC in black women. Another genetic predisposition may be a polymorphism in Lewis blood group antigens that leads to the blood group ABO-Lewis non-secretor phenotype. This status is detected with increased frequency among women with RVVC [281,282].

Genetic polymorphisms in innate immunity genes have been also associated with increased susceptibility to VVC and especially to RVVC, including polymorphisms in MBL gene, dectin-1 stop-codon, Interleukin-4 (IL-4) gene and NLRP3 gene [229]. *Candida* components are initially recognized by the innate immune system through recognition receptors, among which dectin-1 and MBL.

Dectin-1 is a C-type lectin receptor expressed on the cell-membrane of innate immune cells, that amplifies TLR-induced cytokine production (e.g. IL-17, IL-6 and IL-10) [283] . It was detected a mutation in an early stop-codon of dectin-1 gene that leads to the loss of the last nine amino acids of the CDR (carbohydrate recognition domain). This mutation results in the loss of β-glucan recognition and impaired cytokine response, particularly of pro-inflammatory cytokine IL-17[284]. This mutation has been described in a family of patients with RVVC and onycomycosis [223] and seems to have no correlation with susceptibility to systemic candidiasis[285].

Regarding MBL, it is a circulating protein that binds strongly to mannose and N-acetyl-glucosamine residues on the surface of *Candida* microorganisms, activating the complement system

and promoting opsonization [286]. Reduced levels of MBL and increased occurrence of genetic polymorphisms in the MBL gene were found in Chinese and Latvian women with RVVC [224,225,287]. The decrease in circulating MBL concentrations has been shown to correlate with mutations in exon 1 of MBL gene, specially the substitution of an adenine for a guanine in codon 54, which results in impaired assembly and stability of the final MBL protein [224]. The stimulation of *Candida* recognition receptors induces the production of several cytokines, among which the anti-inflammatory IL-4 and the pro-inflammatory Interleukin-1β (IL-1β). IL-4 inhibits anti-*Candida* activity of NO, which is an important effector molecule of the innate immune defence against *Candida* [250]. Thus, high production capacity of IL-4 is associated with immunosuppressive effects. It has been shown that a polymorphism in the promoter region of the IL-4 gene, consisting of a cytosine to thymine variation (C589T), leads to an increased production of IL-4 by immune cells [288]. The occurrence of this IL-4 gene polymorphism has been correlated with high prevalence of RVVC in some studies [287,289]. Furthermore, higher concentrations of vaginal IL-4 and lower vaginal NO concentrations has been reported in patients with RVVC, compared to healthy women [229]. Regarding IL-1β, this cytokine is processed and activated by a complex called inflammasome, which contains the NLR receptors NLRP3, ASC and caspase-1 [229]. Polymorphisms of the NRLP3 gene have been described and seem to have a possibly relationship with RVVC, however this potential association has not been completely clarified [290].

1.5.2 Behavioural factors

1.5.2.1 Contraceptives (oral pills, intrauterine devices and spermicides)

The use of oral contraceptive pills (OCP) has been proposed as a risk factor for VVC by some authors, however, the recognition of this contraceptive method as a risk factor is still controversial because epidemiologic data are contradictory. In fact, some studies reported higher incidence of VVC (39-58% vs 20-38%) [45,139,291] and vaginal colonization by *Candida* (58-69% vs 35-40%) [214,292], in women taking OCP than in patients not using this method. However, other investigators did not find an association between OCP use and vaginal colonization or presence of VVC [40,293,294]. Nevertheless, some authors report that although they did not find increased sporadic VVC in women taking OCP, they found and increased prevalence of RVVC in those women, especially in long time users, proposing OCP as a predisposing factor for RVVC [178,295,296]. The use of OCP causes

anovulation, with the subsequent absence of estrogen and progesterone peaks, usually present in the normal menstrual cycle, but add constant levels of hormones, which may act in the host and in *Candida* microorganisms. Almost all OCP are combinations of synthetic estrogens, as ethinyl estradiol and mestranol, and progestins, as norethindrone, norethynodrel, etynodiol and norgestrel [155]. Some authors believe that the hormonal effects that contribute to VVC in pregnancy are similar to those that act in women taking OCP, especially OCP containing a high hormonal dose [140]. In fact, Cheng et al. [167] detected a similar effect of synthetic ethinyl estradiol and human β-estradiol in the development of *C. albicans* hyphal forms. Furthermore, OCP use may also influence immune responses resulting in decreased vaginal resistance to *Candida*. Levels of lactoferrin and immunoglobulins (IgA and IgG) were shown to be suppressed in women taking OCP for the duration of hormone exposure [297]. Although the hormonal dependency of VVC in pregnancy is well accepted, there is not a consensual acceptation that OCP use is a risk factor for VVC.

The use of intrauterine devices (IUDs) is a highly effective, safe and economic contraception method [76]. It is a very popular way of preventing pregnancy, used by millions of women worldwide and the most preferred mean of contraception in women of developing countries [94,298]. The IUDs are available in several shapes and sizes and are made up of a variety of materials ranging from copper to plastic [299]. The insertion of an IUD stimulates foreign body response or inflammatory reaction, which cause cellular/biochemical alterations in the endometrial mucosa and cervical mucus, leading to the contraceptive effects [94,299]. IUDs that constantly release small amounts of hormones into the uterus are also available [300]. Despite being effective in preventing fertilization, some studies have shown that IUD use increases the risk of complications such as pelvic inflammations, menstrual cramps [299] and, more importantly, infections associated with microbial colonization on these implanted devices, including VVC [94,299]. Although some researchers did not find an association between VVC and IUD use [27,301], it has been proposed as an important risk factor for sporadic VVC and recurrent episodes by many others [25,26,178,179]. The association of IUD use with VVC episodes is potentially due to the *Candida* adhesion and biofilm formation on the surface of the device [93]. The presence of a biofilm on IUD not only protects yeasts from the action of antifungal agents as contributes to the microorganisms' persistence, constituting a source of infection or reinfection [102,302]. Some studies have shown that IUDs recovered from patients suffering from VVC or RVVC are infected with *Candida* biofilms composed of one or multiple species (including bacteria), indicating that the presence of the biofilms on the device possibly contributed to the infection [94,299,302,303]. Importantly, Kamal et al. [303] reported that NCAC species represent more than half of the *Candida* biofilm found in infected

IUDs. Furthermore, Auler et al. [302] verified through electron scanning microscopy that most microorganisms of biofilms formed on IUDs of patients with RVVC had been present on those surfaces for possibly a long time, suggesting that biofilms served as reservoirs of yeasts. Additionally, IUDs may affect the cervico-vaginal microflora by reducing lactobacilli and also modify or destroy the cervical mucus, which serves as a powerful barrier for ascending infections [76]. The IUD removal is required in many infected patients, especially in women with RVVC, in which the removal of the device has been shown to be essential to avoid the relapses [302]. As such, some investigators advise a limited use of the IUDs and a regular check of possible infections during its usage to prevent serious complications [76,179,302].

The use of spermicides and/or condoms has been also associated with increased risk of VVC and RVVC in some studies [26,45,149,176]. This association has been proposed to be due to the active spermicidal compound nonoxynol-9 (N-9), used in spermicides formulations and condoms [304]. N-9 is a non-ionic detergent that immobilizes sperm by disrupting the cell membrane and acts similarly on several pathogens, mainly bacteria and viruses, decreasing the risk of acquiring several sexually transmitted diseases [305]. However, this compound inhibits vaginal commensal organisms, specifically *Lactobacillus* species [304,306], and also causes vaginal epithelium disruption, leading to genital irritation [307,308]. In addition, McGroarty et al. [309] reported that *Candida* microorganisms have the ability to metabolize the N-9 compound, resulting in altered surface characteristics that increase *Candida* adhesion to epithelial tissues. These investigators proposed that N-9, which is a detergent molecule composed of a polar head and a hydrophobic tail, may enhance the adhesion of *Candida* organisms by increasing their hydrophobicity or epithelial cells' hydrophobicity. Despite the use of N-9 in condoms appear to be the most probable cause of their association with VVC development, other reasons have been also proposed such as, allergic reactions to latex, sensitivity of vaginal epithelial cells to condom and vaginal micro traumas with consequent disturbance of the vaginal ecosystem [310,311].

1.5.2.2 Sexual, clothing and hygienic habits

Some sexual practices have been proposed as potential risk factors for VVC, including high frequency of sexual intercourse, which have been shown to be associated with increased vaginal colonization by *Candida* [149], VVC development [312] and recurrent episodes [178,296]. Although VVC is not considered a sexually-transmitted disease, some studies have confirmed *Candida* sexual

transmission [51,203], which may lead to an increase of vaginal *Candida* load, possibly contributing to VVC episodes. Additionally, frequent intercourse may cause vulvovaginal micro-lacerations that create conditions suitable for yeasts to invade the tissue [312,313] and lead to high exposition to numerous antigens, antibodies and cytokines secreted in the seminal fluid, possibly influencing women immune reaction to *Candida* [293]. Furthermore, deposition of semen (alkaline pH) in the vaginal epithelium can lead to an alteration of vaginal microbiota, local mucosa stimulation and introduction of strange bacteria to the vaginal environment [310].

Besides frequent sexual intercourse, also receptive oral intercourse has been proposed as a risk factor for both sporadic [47,293,295] and recurrent VVC episodes [313–315]. It has been shown that *Candida* organisms can be transmitted from the oral cavity, in which they are present in one-third to one-half of adult population, leading to increased vaginal load. Additionally, the saliva may facilitate *Candida* adherence and growth through moistening and irritation of the vaginal mucosa or by changing the local immune responses [176,293]. Other sexual practices such as receptive anal sex [47,313], early sexual debut, casual sex partners [313,316], new sex partner [296], high number of lifetime sex partners [178,316] and sex during menses [313] have been also positively associated with VVC episodes by some studies, although these association is not consensual [293,312].

Some habits of feminine clothing as the use of tight, poorly ventilated and/or synthetic underwear have been also associated with VVC and RVVC development by some studies [53,138,317]. Importantly, a recent study found 100% of vaginal colonization by *Candida* organisms among women that use tight underwear, particularly of nylon, whereas only 38% of women that never use this type of clothing were colonized, suggesting that the use of well-ventilated and cotton underwear could be of value in preventing VVC [318]. It has been suggested that poorly ventilated or synthetic underwear increase the perineal moisture levels and temperature, and may cause local allergy and hypersensitivity reactions, potentially contributing to *Candida* proliferation [12,319]. In fact, feminine hygiene practices that may trigger allergic reactions, as the use of pantyliners [138] and vaginal douching [47,53,316,320], have been also proposed as risk factors for VVC and RVVC development. It is believed that vaginal douching not only introduces exogenous substances that may cause allergic reactions and pH alteration but also promote mechanical cleaning of commensal bacteria, affecting the ecological balance of the vaginal cavity [194]. In addition, it has been recommended for women to avoid excessive washing of vulvar area and potential irritants such as perfumed soaps, bubble baths, powders or vaginal sprays, which also affect the vaginal microbiota [321]. Nevertheless, an unsatisfactory personal hygiene has been also proposed as potential risk for VVC development by some studies [35,322], as it may lead

to an increased number of *Candida* spores in the vaginal environment, due the gut source [317]. However, some authors reported a minor role of intestinal tract in VVC development [20,203] and thus, this association has to be further studied.

Despite research advances, there are still a number of mechanisms involved in VVC development that need to be clarified. The high incidence of VVC, it negatives consequences and the increase of antifungal failure in its treatment, make it crucial to further increase our knowledge on *Candida* vaginal pathogenicity and its underlying mechanisms. Studies in this area will lead to a better understanding of VVC, contributing to the identification of new targets for more efficient therapeutic approaches against this clinically relevant fungal infection.

References

[1] **Sidrim J, Rocha M**. Biologia dos Fungos. *Micol. Médica à luz autores Contemp.* Guanabara Koogan; (2004). pp. 82–8.

[2] **Dignani M, Solomkin J, Anaissie E**. *Candida*. *Clin. Mycol.* Churchill Livingstone; (2009). pp. 197–231.

[3] **Ascioglu S, Rex JH, de Pauw B, et al.** Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* (2002); 34(1):7–14.

[4] **Beigi RH, Meyn LA, Moore DM, et al.** Vaginal yeast colonization in nonpregnant women: a longitudinal study. *Obstet Gynecol* (2004); 104(5 Pt 1):926–30.

[5] **Bertholf ME, Stafford MJ**. Colonization of *Candida albicans* in vagina, rectum, and mouth. *J Fam Pract* (1983); 16(5):919–24.

[6] **Ferrer J**. Vaginal candidosis: epidemiological and etiological factors. *Int J Gynecol Obstet* (2000); 71:21–7.

[7] **Achkar JM, Fries BC**. *Candida* infections of the genitourinary tract. *Clin Microbiol Rev* (2010); 23(2):253–73.

[8] **Pappas PG, Kauffman C a, Andes D, et al.** Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* (2009); 48(5):503–35.

[9] **Sobel JD, Faro S, Force RW, et al.** Vulvovaginal candidiasis: Epidemiologic, diagnostic, and therapeutic considerations. *Am J Obstet Gynecol* (1998); 178(2):203–11.

[10] **Anderson MR**. Evaluation of Vaginal Complaints. *JAMA* (2004); 291(11):1368–79.

[11] **Sobel JD**. Genital candidiasis. *Medicine (Baltimore)* (2005); 33(10):62–5.

[12] **Sobel JD**. Genital candidiasis. *Medicine (Baltimore)* (2014); 38(6):186–90.

[13] **Watson MC, Grimshaw JM, Bond CM, et al.** Oral versus intra-vaginal imidazole and triazole anti-fungal agents for the treatment of uncomplicated vulvovaginal candidiasis (thrush): a systematic review. *BJOG* (2002); 109(1):85–95.

[14] **Palmeira-de-Oliveira R, Duarte P, Palmeira-de-Oliveira A, et al.** Women's experiences, preferences and perceptions regarding vaginal products: Results from a cross-sectional web-based survey in Portugal. *Eur J Contracept Reprod Heal Care* (2015); 20(4):259–71.

[15] **Fidel PL, Vazquez JA, Sobel JD**. *Candida glabrata* : review of epidemiology, pathogenesis, and clinical disease

with comparison to *C. albicans*. *Clin Microbiol Rev* (1999); 12(1):80–96.

[16] **Sobel JD, Sobel R**. Current treatment options for vulvovaginal candidiasis caused by azole-resistant *Candida* species. *Expert Opin Pharmacother* (2018); 19(9):971–7.

[17] **Petersen KA, Matthiesen F, Agger T, et al.** Phase I safety, tolerability, and pharmacokinetic study of recombinant human mannan-binding lectin. *J Clin Immunol* (2006); 26(5):465–75.

[18] **Spellberg BJ, Ibrahim AS, Avanesian V, et al.** Efficacy of the anti- *Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J Infect Dis* (2006); 194(2):256–60.

[19] **Pericolini E, Gabrielli E, Amacker M, et al.** Secretory aspartyl proteinases cause vaginitis and can mediate vaginitis caused by *Candida albicans* in mice. *MBio* (2015); 6(3):e00724.

[20] **Sobel JD**. Vulvovaginal candidosis. *Lancet* (2007); 369(9577):1961–71.

[21] **Foxman B, Barlow R, D'Arcy H, et al.** *Candida* vaginitis: self-reported incidence and associated costs. *Sex Transm Dis* (2000); 27(4):230–5.

[22] **Røttingen JA, Cameron DW, Garnett GP**. A systematic review of the epidemiologic interactions between classic sexually transmitted diseases and HIV: how much really is known? *Sex Transm Dis* (2001); 28(10):579–97.

[23] **Nwadioha SI, Egah DZ, Alao OO, et al.** Risk factors for vaginal candidiasis among women attending primary health care centers of Jos , Nigeria. *J Clin Med Res* (2010); 2:110–3.

[24] **Emeride A, Abdullahi Nasir I, Uchenna E, et al.** Prevalence of vulvovaginal candidiasis among nonpregnant women attending a tertiary health care facility in Abuja, Nigeria. *Res Rep Trop Med* (2015); 6:37.

[25] **Kamal AD, Hassan SAEK, Nawar NN, et al.** Prevalence of common types of vaginal infections among women attending gynaecology clinics in Egypt. *Malaysian J Med Res* (2019); 3(1):41–9.

[26] **Amouri I, Sellami H, Borji N, et al.** Epidemiological survey of vulvovaginal candidosis in Sfax, Tunisia. *Mycoses* (2011); 54(5):e499-505.

[27] **Mtibaa L, Fakhfakh N, Kallel A, et al.** Vulvovaginal candidiasis: Etiology, symptomatology and risk factors. *J Mycol Med* (2017); 27(2):153–8.

[28] **Gharaghani M, Ahmadi B, Taheripour Sisakht M, et al.** Identification of *Candida* species isolated from vulvovaginal candidiasis patients by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in Yasuj southwestern Iran. *Jundishapur J Microbiol* (2018); 11(8).

[29] **Hedayati MT, Taheri Z, Galinimoghadam T, et al.** Isolation of different species of *Candida* in patients with vulvovaginal candidiasis from sari, iran. *Jundishapur J Microbiol* (2015); 8(4):e15992.

[30] **Rezaei-Matehkolaei A, Shafiei S, Zarei-Mahmoudabadi A**. Isolation, molecular identification, and antifungal susceptibility profiles of vaginal isolates of *Candida* species. *Iran J Microbiol* (2016); 8(6):410–7.

[31] **Nabi SN, Rahimgir M, Khan AA, et al.** Epidemiologic features of vulvovaginal candidiasis in women of childbearing age in a combined military hospital. *J Armed Forces Med Coll Bangladesh* (2017); 13(1):13–6.

[32] **Kapoor A, Kapoor B, Shah S, et al.** Epidemiological study in symptomatic patients of vaginal discharge in industrial population. *J Evol Med Dent Sci* (2016); 5(01):92–5.

[33] **Krishnasamy L, Krishnakumar S, Santharam P, et al.** Isolation and identification of *Candida* species in patients with vulvovaginal candidiasis. *J Pure Appl Microbiol* (2018); 12(4):2269–73.

[34] **Mohanty S, Xess I, Hasan F, et al.** Prevalence & susceptibility to fluconazole of *Candida* species causing vulvovaginitis. *Indian J Med Res* (2007); 126(3):216–9.

[35] **Ahmad A, Khan AU**. Prevalence of *Candida* species and potential risk factors for vulvovaginal candidiasis in Aligarh, India. *Eur J Obstet Gynecol Reprod Biol* (2009); 144(1):68–71.

[36] **Vijaya D, Dhanalakshmi TA, Kulkarni S**. Changing trends of vulvovaginal candidiasis. *J Lab Physicians* (2014); 6(1):28–30.

[37] **Kandel S, Shrestha R, Adhikary P**. Study of prevalence of *Candida albicans* among the patients attending to out patient services og gynaecology and obstetrics departament with complaint of vaginal discharge. *World J Pharm Pharm Sci* (2017); 6(2):1457–63.

[38] **Shi X-Y, Yang Y-P, Zhang Y, et al.** Molecular identification and antifungal susceptibility of 186 *Candida* isolates from vulvovaginal candidiasis in southern China. *J Med Microbiol* (2015); 64(Pt_4):390–3.

[39] **Rosa MI da, Rumel D**. Fatores associados à candidíase vulvovaginal: estudo exploratório. *Rev Bras Ginecol e Obs* (2004); 26(1):65–70.

[40] **Andrioli JL, Oliveira GSA, Barreto CS, et al.** Frequência de leveduras em fluido vaginal de mulheres com e sem suspeita clínica de candidíase vulvovaginal. *Rev Bras Ginecol e Obs* (2009); 31(6):300–4.

[41] **Jackson S, Mullings A, Rainford L, et al.** The epidemiology of mycotic vulvovaginitis and the use of antifungal agents in suspected mycotic vulvovaginitis and its implications for clinical practice. *West Indian Med J* (2005); 54(3).

[42] **Tibaldi C, Cappello N, Latino MA, et al.** Vaginal and endocervical microorganisms in symptomatic and asymptomatic non-pregnant females: risk factors and rates of occurrence. *Clin Microbiol Infect* (2009); 15(7):670–9.

[43] **Paulitsch A, Weger W, Ginter-Hanselmayer G, et al.** A 5-year (2000-2004) epidemiological survey of *Candida* and non-*Candida* yeast species causing vulvovaginal candidiasis in Graz, Austria. *Mycoses* (2006); 49(6):471–5.

[44] **Grigoriou O, Baka S, Makrakis E, et al.** Prevalence of clinical vaginal candidiasis in a university hospital and possible risk factors. *Eur J Obstet Gynecol Reprod Biol* (2006); 126(1):121–5.

[45] **Cetin M, Ocak S, Gungoren A, et al.** Distribution of *Candida* species in women with vulvovaginal symptoms and their association with different ages and contraceptive methods. *Scand J Infect Dis* (2007); 39(6–7):584–8.

[46] **Dan M, Kaneti N, Levin D, et al.** Vaginitis in a gynecologic practice in Israel: causes and risk factors. *Isr Med Assoc J* (2003); 5(9):629–32.

[47] **Bradshaw CS, Morton AN, Garland SM, et al.** Higher-risk behavioral practices associated with bacterial vaginosis compared with vaginal candidiasis. *Obstet Gynecol* (2005); 106(1):105–14.

[48] **Mahmoudi Rad M, Zafarghandi S, Abbasabadi B, et al.** The epidemiology of *Candida* species associated with vulvovaginal candidiasis in an Iranian patient population. *Eur J Obstet Gynecol Reprod Biol* (2011); 155(2):199–203.

[49] **Alves CT, Wei X-Q, Silva S, et al.** *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. *J Infect* (2014); 69(4):396–407.

[50] **Richter SS, Galask RP, Messer SA, et al.** Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol* (2005); 43(5):2155–62.

[51] **Boatto HF, Moraes MS de, Machado AP, et al.** Correlação entre os resultados laboratoriais e os sinais e sintomas clínicos das pacientes com candidíase vulvovaginal e relevância dos parceiros sexuais na manutenção da infecção em São Paulo, Brasil. *Rev Bras Ginecol e Obs* (2007); 29(2):80–4.

[52] **Brandolt TM, Klafke GB, Gonçalves CV, et al.** Prevalence of *Candida spp*. in cervical-vaginal samples and the in vitro susceptibility of isolates. *Brazilian J Microbiol* (2017); 48(1):145–50.

[53] **Corsello S, Spinillo A, Osnengo G, et al.** An epidemiological survey of vulvovaginal candidiasis in Italy. *Eur J Obstet Gynecol Reprod Biol* (2003); 110(1):66–72.

[54] **Sojakova M, Liptajova D, Borovsky M, et al.** Fluconazole and itraconazole susceptibility of vaginal yeast isolates from Slovakia. *Mycopathologia* (2004); 157(2):163–9.

[55] **Díaz M, Camponovo R, Araya I, et al.** Identificación y sensibilidad antifúngica in vitro de *Candida spp*. de origen vaginal a fluconazol, clotrimazol y nistatina. *Rev Española Quimioter* (2016); 29(3):151–4.

[56] **Fan SR, Liu XP, Li JW**. Clinical characteristics of vulvovaginal candidiasis and antifungal susceptibilities of *Candida* species isolates among patients in southern China from 2003 to 2006. *J Obstet Gynaecol Res* (2008); 34(4):561–6.

[57] **Okungbowa FI, Isikhuemhen OS, Dede APO**. The distribution frequency of *Candida* species in the genitourinary tract among symptomatic individuals in Nigerian cities. *Rev Iberoam Micol* (2003); 20(2):60–3.

[58] **Holland J, Young ML, Lee O, et al.** Vulvovaginal carriage of yeasts other than *Candida albicans*. *Sex Transm Infect* (2003); 79(3):249–50.

[59] **Linhares IM, Witkin SS, Miranda SD, et al.** Differentiation between women with vulvovaginal symptoms who are positive or negative for *Candida* species by culture. *Infect Dis Obstet Gynecol* (2001); 9(4):221–5.

[60] **Lynch ME, Sobel JD**. Comparative *in vitro* activity of antimycotic agents against pathogenic vaginal yeast isolates. *Med Mycol* (1994); 32(4):267–74.

[61] **Otero L, Palacio V, Carreno F, et al.** Vulvovaginal candidiasis in female sex workers. *Int J STD AIDS* (1998); 9(9):526–30.

[62] **Saporiti AM, Gómez D, Levalle S, et al.** Vaginal candidiasis: etiology and sensitivity profile to antifungal agents in clinical use. *Rev Argent Microbiol* (2001); 33(4):217–22.

[63] **Spinillo A, Capuzzo E, Gulminetti R, et al.** Prevalence of and risk factors for fungal vaginitis caused by non-*albicans* species. *Am J Obstet Gynecol* (1997); 176(1 Pt 1):138–41.

[64] **Nagashima M, Yamagishi Y, Mikamo H**. Antifungal susceptibilities of *Candida* species isolated from the patients with vaginal candidiasis. *J Infect Chemother* (2016); 22(2):124–6.

[65] **Nyirjesy P, Weitz M, Grody M, et al.** Over-the-counter and alternative medicines in the treatment of chronic vaginal symptoms. *Obstet Gynecol* (1997); 90(1):50–3.

[66] **Dan M, Segal R, Marder V, et al.** *Candida* colonization of the vagina in elderly residents of a long-term-care hospital. *Eur J Clin Microbiol Infect Dis* (2006); 25(6):394–6.

[67] **Goswami R, Dadhwal V, Tejaswi S, et al.** Species-specific prevalence of vaginal candidiasis among patients with diabetes mellitus and its relation to their glycaemic status. *J Infect* (2000); 41(2):162–6.

[68] **Goswami D, Goswami R, Banerjee U, et al.** Pattern of *Candida* species isolated from patients with diabetes mellitus and vulvovaginal candidiasis and their response to single dose oral fluconazole therapy. *J Infect* (2006); 52(2):111–7.

[69] **Sobel JD, Chaim W, Nagappan V, et al.** Treatment of vaginitis caused by *Candida glabrata*: use of topical boric acid and flucytosine. *Am J Obstet Gynecol* (2003); 189(5):1297–300.

[70] **Redding SW, Kirkpatrick WR, Saville S, et al.** Multiple patterns of resistance to fluconazole in *Candida glabrata* isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. *J Clin Microbiol* (2003); 41(2):619–22.

[71] **Spitzer M, Wiederhold NP**. Reduced antifungal susceptibility of vulvovaginal *Candida* species at ormal vaginal pH levels. *J Low Genit Tract Dis* (2018); 22(2):152–8.

[72] **Tamura NK, Negri MFN, Bonassoli LA, et al.** Fatores de virulência de *Candida spp* isoladas de cateteres venosos e mãos de servidores hospitalares. *Rev Soc Bras Med Trop* (2007); 40(1):91–3.

[73] **Silva S, Negri M, Henriques M, et al.** *Candida glabrata*, *Candida parapsilosis* and Candida *tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev* (2012); 36(2):288–305.

[74] **Wächtler B, Wilson D, Hube B**. *Candida albicans* adhesion to and invasion and damage of vaginal epithelial cells: stage-specific inhibition by clotrimazole and bifonazole. *Antimicrob Agents Chemother* (2011); 55(9):4436–9.

[75] **Vidotto V, Mantoan B, Pugliese A, et al.** Adherence of *Candida albicans* and *Candida dubliniensis* to buccal and vaginal cells. *Rev Iberoam Micol* (2003); 20(2):52–4.

[76] **Demirezen S, Dirlik OO, Beksaç MS**. The association of *Candida* infection with intrauterine contraceptive device. *Cent Eur J Public Health* (2005); 13(1):32–4.

[77] **Verstrepen KJ, Klis FM**. Flocculation, adhesion and biofilm formation in yeasts. *Mol Microbiol* (2006); 60(1):5–15.

[78] **Chaffin WL**. *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev* (2008); 72(3):495–544.

[79] **Hoyer LL, Green CB, Oh S-H, et al.** Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family–a sticky pursuit. *Med Mycol* (2008); 46(1):1–15.

[80] **Cheng G, Wozniak K, Wallig MA, et al.** Comparison between *Candida albicans* agglutinin-like sequence gene expression patterns in human clinical specimens and models of vaginal candidiasis. *Infect Immun* (2005); 73(3):1656–63.

[81] **Rahimi H, Roudbarmohammadi S, Kachouei R, et al.** Expression of *Candida albicans* ALS 2 and ALS 9 genes isolated from women with vaginal candidiasis by RT-PCR. *Pathobiol Res* (2013); 16(2):39–49.

[82] **Cormack BP**. An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science (80-)* (1999); 285(5427):578–82.

[83] **Butler G, Rasmussen MD, Lin MF, et al.** Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* (2009); 459(7247):657–62.

[84] **Hoyer LL, Fundyga R, Hecht JE, et al.** Characterization of agglutinin-like sequence genes from non-*albicans Candida* and phylogenetic analysis of the ALS family. *Genetics* (2001); 157(4):1555–67.

[85] **Douglas LJ**. *Candida* biofilms and their role in infection. *Trends Microbiol* (2003); 11(1):30–6.

[86] **Donlan RM, Costerton JW**. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* (2002); 15(2):167–93.

[87] **Davey ME, O'toole GA**. Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* (2000); 64(4):847–67.

[88] **Ramage G, Mowat E, Jones B, et al.** Our current understanding of fungal biofilms. *Crit Rev Microbiol* (2009); 35(4):340–55.

[89] **Ramage G, Rajendran R, Sherry L, et al.** Fungal biofilm resistance. *Int J Microbiol* (2012); 2012:1–14.

[90] **Jain N, Kohli R, Cook E, et al.** Biofilm formation by and antifungal susceptibility of *Candida* isolates from urine. *Appl Environ Microbiol* (2007); 73(6):1697–703.

[91] **Silva S, Henriques M, Martins A, et al.** Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* (2009); 47(7):681–9.

[92] **Harriott MM, Lilly EA, Rodriguez TE, et al.** *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology* (2010); 156(Pt 12):3635–44.

[93] **Chassot F, Negri MFN, Svidzinski AE, et al.** Can intrauterine contraceptive devices be a *Candida albicans* reservoir? *Contraception* (2008); 77(5):355–339.

[94] **Lal P, Agarwal V, Pruthi P, et al.** Biofilm formation by *Candida albicans* isolated from intrauterine devices. *Indian J Microbiol* (2008); 48(4):438–44.

[95] **Dominic RM, Shenoy S, Baliga S**. *Candida* biofilms in medical devices: evolving trends. *Kathmandu Univ Med J (KUMJ)* (2007); 5(3):431–6.

[96] **Nobile CJ, Andes DR, Nett JE, et al.** Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathog* (2006); 2(7):e63.

[97] **Ramage G, VandeWalle K, LÃ³pez-Ribot JL, et al.** The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol Lett* (2002); 214(1):95–100.

[98] **Thein ZM, Seneviratne CJ, Samaranayake YH, et al.** Community lifestyle of *Candida* in mixed biofilms: a mini review. *Mycoses* (2009); 52(6):467–75.

[99] **Swidsinski A, Mendling W, Loening-Baucke V, et al.** An adherent Gardnerella vaginalis biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am J Obstet Gynecol* (2008); 198(1):97.e1-97.e6.

[100] **Špaček J, Buchta V, Jílek P, et al.** Clinical aspects and luteal phase assessment in patients with recurrent vulvovaginal candidiasis. *Eur J Obstet Gynecol Reprod Biol* (2007); 131(2):198–202.

[101] **Esim Buyukbayrak E, Kars B, Karsidag AYK, et al.** Diagnosis of vulvovaginitis: comparison of clinical and microbiological diagnosis. *Arch Gynecol Obstet* (2010); 282(5):515–9.

[102] **Pal Z, Urbán E, Dósa E, et al.** Biofilm formation on intrauterine devices in relation to duration of use. *J Med Microbiol* (2005); 54(12):1199–203.

[103] **Rivers CA, Adaramola OO, Schwebke JR**. Prevalence of bacterial vaginosis and vulvovaginal candidiasis mixed infection in a southeastern American STD clinic. *Sex Transm Dis* (2011); 38(7):672–4.

[104] **Bradshaw CS, Morton AN, Hocking J, et al.** High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. *J Infect Dis* (2006); 193(11):1478–86.

[105] **McClelland RS, Richardson BA, Hassan WM, et al.** Prospective study of vaginal bacterial flora and other risk factors for vulvovaginal candidiasis. *J Infect Dis* (2009); 199(12):1883–90.

[106] **Schaller M, Borelli C, Korting HC, et al.** Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* (2005); 48(6):365–77.

[107] **Pichová I, Pavlíčková L, Dostál J, et al.** Secreted aspartic proteases of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida lusitaniae*. *Eur J Biochem* (2001); 268(9):2669–77.

[108] **Odds FC**. Secreted proteinases and *Candida albicans* virulence. *Microbiology* (2008); 154(11):3245–6.

[109] **Merkerová M, Dostál J, Hradilek M, et al.** Cloning and characterization of Sapp2p, the second aspartic proteinase isoenzyme from *Candida parapsilosis*. *FEMS Yeast Res* (2006); 6(7):1018–26.

[110] **Togni G, Sanglard D, Falchetto R, et al.** Isolation and nucleotide sequence of the extracellular acid protease gene (ACP) from the yeast *Candida tropicalis*. *FEBS Lett* (1991); 286(1–2):181–5.

[111] **Zaugg C, Borg-von Zepelin M, Reichard U, et al.** Secreted aspartic proteinase family of *Candida tropicalis*. *Infect Immun* (2001); 69(1):405–12.

[112] **Kaur R, Ma B, Cormack BP**. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci* (2007); 104(18):7628–33.

[113] **Bairwa G, Kaur R**. A novel role for a glycosylphosphatidylinositol-anchored aspartyl protease, CgYps1, in the regulation of pH homeostasis in *Candida glabrata*. *Mol Microbiol* (2011); 79(4):900–13.

[114] **Williams DW, Kuriyama T, Silva S, et al.** *Candida* biofilms and oral candidosis: treatment and prevention. *Periodontol 2000* (2011); 55(1):250–65.

[115] **Mohandas V, Ballal M**. Distribution of *Candida* species in different clinical samples and their virulence: biofilm formation, proteinase and phospholipase production: a study on hospitalized patients in southern India. *J Glob Infect Dis* (2011); 3(1):4–8.

[116] **Lima JS, Braga KRGS, Vieira CA, et al.** Genotypic analysis of secreted aspartyl proteinases in vaginal *Candida albicans* isolates. *J Bras Patol e Med Lab* (2018); 54(1):28–33.

[117] **Ozcan SK, Budak F, Yucesoy G, et al.** Prevalence, susceptibility profile and proteinase production of yeasts causing vulvovaginitis in Turkish women. *APMIS* (2006); 114(2):139–45.

[118] **Lian CH, Liu W Da**. Differential expression of *Candida albicans* secreted aspartyl proteinase in human vulvovaginal candidiasis. *Mycoses* (2007); 50(5):383–90.

[119] **Naglik JR, Rodgers CA, Shirlaw PJ, et al.** Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *J Infect Dis* (2003); 188(3):469–79.

[120] **Monroy-Pérez E, Paniagua-Contreras G, Vaca-Paniagua F, et al.** SAP expression in *Candida albicans* strains isolated from mexican patients with vaginal candidosis. *Int J Clin Med* (2013); 04(01):25–31.

[121] **Ghannoum MA**. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* (2000); 13(1):122–43.

[122] **Samaranayake YH, Dassanayake RS, Cheung BPK, et al.** Differential phospholipase gene expression by *Candida albicans* in artificial media and cultured human oral epithelium. *APMIS* (2006); 114(12):857–66.

[123] **Stehr F, Felk A, Gacser A, et al.** Expression analysis of the *Candida* albicans lipase gene family during experimental infections and in patient samples. *FEMS Yeast Res* (2004); 4(4–5):401–8.

[124] **Luo G, Samaranayake LP, Cheung BPK, et al.** Reverse transcriptase polymerase chain reaction (RT-PCR) detection of HLP gene expression in *Candida glabrata* and its possible role in in vitro haemolysin production. *APMIS* (2004); 112(4–5):283–90.

[125] **Kumamoto CA, Vinces MD**. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell Microbiol* (2005); 7(11):1546–54.

[126] **Hostetter MK**. Adhesins and ligands involved in the interaction of *Candida spp*. with epithelial and endothelial surfaces. *Clin Microbiol Rev* (1994); 7(1):29–42.

[127] **Hube B, Sanglard D, Odds FC, et al.** Disruption of each of the secreted aspartyl proteinase genes SAP1, SAP2, and SAP3 of *Candida albicans* attenuates virulence. *Infect Immun* (1997); 65(9):3529–38.

[128] **Taylor BN, Staib P, Binder A, et al.** Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. *Infect Immun* (2005); 73(3):1828–35.

[129] **Anderson JM, Soll DR**. Unique phenotype of opaque cells in the white-opaque transition of *Candida albicans*. *J Bacteriol* (1987); 169(12):5579–88.

[130] **Zordan RE, Galgoczy DJ, Johnson AD**. Epigenetic properties of white-opaque switching in *Candida albicans* are based on a self-sustaining transcriptional feedback loop. *Proc Natl Acad Sci* (2006); 103(34):12807–12.

[131] **Zordan RE, Miller MG, Galgoczy DJ, et al.** Interlocking transcriptional feedback loops control white-opaque switching in *Candida albicans*. *PLoS Biol* (2007); 5(10):e256.

[132] **Soll DR**. Gene regulation during high-frequency switching in *Candida albicans*. *Microbiology* (1997); 143(2):279–88.

[133] **Brockert PJ, Lachke SA, Srikantha T, et al.** Phenotypic switching and mating type switching of *Candida glabrata* at sites of colonization. *Infect Immun* (2003); 71(12):7109–18.

[134] **Vargas K, Messer SA, Pfaller M, et al.** Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. *J Clin Microbiol* (2000); 38(10):3595–607.

[135] **Morrow B, Srikantha T, Soll DR**. Transcription of the gene for a pepsinogen, PEP1, is regulated by white-opaque switching in *Candida albicans*. *Mol Cell Biol* (1992); 12(7):2997–3005.

[136] **Soll DR**. High-frequency switching in *Candida albicans* and its relations to vaginal candidiasis. *Am J Obstet Gynecol* (1988); 158(4):997–1001.

[137] **Soll DR, Galask R, Isley S, et al.** Switching of *Candida albicans* during successive episodes of recurrent vaginitis. *J Clin Microbiol* (1989); 27(4):681–90.

[138] **Patel DA, Gillespie B, Sobel JD, et al.** Risk factors for recurrent vulvovaginal candidiasis in women receiving maintenance antifungal therapy: results of a prospective cohort study. *Am J Obstet Gynecol* (2004); 190(3):644–53.

[139] **Neerja J, Aruna A, Paramjeet G**. Significance of *Candida* culture in women with vulvovaginal symptoms. *J Obstet Gynecol India* (2006); 56(2):139–41.

[140] **Bauters TGM, Dhont MA, Temmerman MIL, et al.** Prevalence of vulvovaginal candidiasis and susceptibility to fluconazole in women. *Am J Obstet Gynecol* (2002); 187(3):569–74.

[141] **Kamath P, Pais M, Nayak MG**. Risk of vaginal candidiasis among pregnant women. *Int J Curr Microbiol Appl Sci* (2013); 2(9):141–6.

[142] **Shu'aibu I, Tennu P, Fatima U, et al.** Incidence of vulvovaginal candidiasis associated with *Candida albicans* among pregnant women attending Gombe State Specialist Hospital, Gombe, Nigeria. *Bayero J Pure Appl Sci* (2014); 6(2):84.

[143] **Nelson M, Wanjiru W, Margaret MW**. Prevalence of vaginal candidiasis and determination of the occurrence of *Candida* species in pregnant women attending the antenatal clinic of Thika district Hospital, Kenya. *Open J Med Microbiol* (2013); 03(04):264–72.

[144] **Sangaré I, Sirima C, Bamba S, et al.** Prevalence of vulvovaginal candidiasis in pregnancy at three health centers in Burkina Faso. *J Mycol Med* (2018); 28(1):186–92.

[145] **Yadav K, Prakash S**. Prevalence of vulvovaginal candidiasis in pregnancy. *Glob J Med Med Sci* (2016); 4(1):108–16.

[146] **Cotch MF, Hillier SL, Gibbs RS, et al.** Epidemiology and outcomes associated with moderate to heavy *Candida* colonization during pregnancy. *Am J Obstet Gynecol* (1998); 178(2):374–80.

[147] **Jabeen R, Siddiqi I**. Frequency of Vaginal Candidiasis amongst Pregnant Women & Effect of Predisposing

Factors. *Int Ophthalmol Updat* (2014); 12(2):140–3.

[148] **Rai M, Poudel TP, Gurung K, et al.** Prevalence of *Candida albicans* in genital tract of pregnant women attending antenatal clinic of Nepalgunj Medical College Hospital. *J Nepalgunj Med Coll* (2017); 15(2):13–7.

[149] **Eckert L**. Vulvovaginal candidiasis: clinical manifestations, risk factors, management algorithm. *Obstet Gynecol* (1998); 92(5):757–65.

[150] **Kalo A, Segal E**. Interaction of *Candida albicans* with genital mucosa: effect of sex hormones on adherence of yeasts in vitro. *Can J Microbiol* (1988); 34(3):224–8.

[151] **McNagny SE**. Prescribing hormone replacement therapy for menopausal symptoms. *Ann Intern Med* (1999); 131(8):605–16.

[152] **Psaty BM**. A review of the association of estrogens and progestins with cardiovascular disease in postmenopausal women. *Arch Intern Med* (1993); 153(12):1421–7.

[153] **Dennerstein GJ, Ellis DH**. Oestrogen, glycogen and vaginal candidiasis. *Aust New Zeal J Obstet Gynaecol* (2001); 41(3):326–8.

[154] **Spinillo A, Bernuzzi AM, Cevini C, et al.** The relationship of bacterial vaginosis, *Candida* and trichomonas infection to symptomatic vaginitis in postmenopausal women attending a vaginitis clinic. *Maturitas* (1997); 27(3):253–60.

[155] **Guyton A, Hall J**. Female physiology before pregnancy and female homones. *Textb. Med. Physiol.* 11th edn. Amsterdam: Elsevier; (2006). pp. 1011–26.

[156] **Kalo-Klein A, Witkin SS**. Regulation of the immune response to *Candida albicans* monocytes and progesterone. *Am J Obstet Gynecol* (1991); 164(5):1351–4.

[157] **Wira CR, Fahey J V**. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. *AIDS* (2008); 22(15):1909–17.

[158] **Keller MJ, Guzman E, Hazrati E, et al.** PRO 2000 elicits a decline in genital tract immune mediators without compromising intrinsic antimicrobial activity. *AIDS* (2007); 21(4):467–76.

[159] **Nohmi T, Abe S, Dobashi K, et al.** Suppression of anti- *Candida* activity of murine neutrophils by progesterone in vitro : a possible mechanism in pregnant women's vulnerability to vaginal candidiasis. *Microbiol Immunol* (1995); 39(6):405–9.

[160] **Wagner RD, Johnson SJ**. Probiotic lactobacillus and estrogen effects on vaginal epithelial gene expression responses to *Candida albicans*. *J Biomed Sci* (2012); 19:58.

[161] **Powell BL, Frey CL, Drutz DJ**. Identification of a 17β-estradiol binding protein in *Candida albicans* and *Candida* (Torulopsis) *glabrata*. *Exp Mycol* (1984); 8(4):304–13.

[162] **Malloy PJ, Zhao X, Madani ND, et al.** Cloning and expression of the gene from *Candida albicans* that encodes a high-affinity corticosteroid-binding protein. *Proc Natl Acad Sci* (1993); 90(5):1902–6.

[163] **Loose DS, Stevens D a, Schurman DJ, et al.** Distribution of a corticosteroid-binding protein in *Candida* and other fungal genera. *J Gen Microbiol* (1983); 129(8):2379–85.

[164] **Alves CT, Silva S, Pereira L, et al.** Effect of progesterone on *Candida albicans* vaginal pathogenicity. *Int J Med Microbiol* (2014); 304(8):1011–7.

[165] **Banerjee D, Martin N, Nandi S, et al.** A genome-wide steroid response study of the major human fungal pathogen *Candida albicans*. *Mycopathologia* (2007); 164(1):1–17.

[166] **Gujjar PR, Finucane M, Larsen B**. The effect of estradiol on *Candida albicans* growth. *Ann Clin Lab Sci* n.d.; 27(2):151–6.

[167] **Cheng G, Yeater KM, Hoyer LL**. Cellular and molecular biology of *Candida albicans* estrogen response. *Eukaryot Cell* (2006); 5(1):180–91.

[168] **Kurakado S, Kurogane R, Sugita T**. 17β-Estradiol inhibits estrogen binding protein-mediated hypha formation in *Candida albicans*. *Microb Pathog* (2017); 109:151–5.

[169] **O'Connor C, Essmann M, Larsen B**. 17-β-estradiol upregulates the stress response in *Candida albicans*:

implications for microbial virulence. *Infect Dis Obstet Gynecol* (1998); 6(4):176–81.

[170] **Aguin TJ, Sobel JD**. Vulvovaginal candidiasis in pregnancy. *Curr Infect Dis Rep* (2015); 17(6):30.

[171] **Parveen N, Munir AA, Din I, et al.** Frequency of vaginal candidiasis in pregnant women attending routine antenatal clinic. *J Coll Physicians Surg Pak* (2008); 18(3):154–7.

[172] **Czeizel, Rockenbauer**. A lower rate of preterm birth after clotrimazole therapy during pregnancy. *Paediatr Perinat Epidemiol* (1999); 13(1):58–64.

[173] **Czeizel AE, Fladung B, Vargha P**. Preterm birth reduction after clotrimazole treatment during pregnancy. *Eur J Obstet Gynecol Reprod Biol* (2004); 116(2):157–63.

[174] **Mitchell H**. Vaginal discharge–causes, diagnosis, and treatment. *BMJ* (2004); 328(7451):1306–8.

[175] **Barbone F, Austin H, Louv WC, et al.** A follow-up study of methods of contraception, sexual activity, and rates of trichomoniasis, candidiasis, and bacterial vaginosis. *Am J Obstet Gynecol* (1990); 163(2):510–4.

[176] **Geiger AM, Foxman B**. Risk factors for vulvovaginal candidiasis: a case-control study among university students. *Epidemiology* (1996); 7(2):182–7.

[177] **Spinillo A, Capuzzo E, Acciano S, et al.** Effect of antibiotic use on the prevalence of symptomatic vulvovaginal candidiasis. *Am J Obstet Gynecol* (1999); 180(1):14–7.

[178] **Spinillo A, Capuzzo E, Nicola S, et al.** The impact of oral contraception on vulvovaginal candidiasis. *Contraception* (1995); 51(5):293–7.

[179] **Guzel AB, Ilkit M, Akar T, et al.** Evaluation of risk factors in patients with vulvovaginal candidiasis and the value of chromID *Candida* agar versus CHROMagar *Candida* for recovery and presumptive identification of vaginal yeast species. *Med Mycol* (2011); 49(1):16–25.

[180] **Bluestein D, Rutledge C, Lumsden L**. Predicting the occurrence of antibiotic-induced candidal vaginitis (AICV). *Fam Pract Res J* (1991); 11(3):319–26.

[181] **Morales DK, Hogan DA**. *Candida albicans* interactions with bacteria in the context of human health and disease. *PLoS Pathog* (2010); 6(4):e1000886.

[182] **Pirotta M V, Garland SM**. Genital *Candida* species detected in samples from women in Melbourne, Australia, before and after treatment with antibiotics. *J Clin Microbiol* (2006); 44(9):3213–7.

[183] **Xu J, Schwartz K, Bartoces M, et al.** Effect of antibiotics on vulvovaginal candidiasis: a MetroNet study. *J Am Board Fam Med* (2008); 21(4):261–8.

[184] **Hamad M, Muta'eb E, Abu-Shaqra Q, et al.** Utility of the oestrogen-dependent vaginal candidosis murine model in evaluating the efficacy of various therapies against vaginal *Candida albicans* infection. *Mycoses* (2006); 49(2):104–8.

[185] **Cribby S, Taylor M, Reid G**. Vaginal microbiota and the use of probiotics. *Interdiscip Perspect Infect Dis* (2008); 2008:1–9.

[186] **Boris S, Barbés C**. Role played by lactobacilli in controlling the population of vaginal pathogens. *Microbes Infect* (2000); 2(5):543–6.

[187] **Boris S, Suarez JE, Vazquez F, et al.** Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect Immun* (1998); 66(5):1985–9.

[188] **Osset J, García E, Bartolomé RM, et al.** Role of Lactobacillus as protector against vaginal candidiasis. *Med Clin (Barc)* (2001); 117(8):285–8.

[189] **Velraeds MMC, Van De Belt-Gritter B, Van Der Mei HC, et al.** Interference in initial Adhesion of uropathogenic bacteria and yeasts to silicone rubber by a Lactobacillus acidophilus biosurfactant. *J Med Microbiol* (1998); 47(12):1081–5.

[190] **Okkers DJ, Dicks LMT, Silvester M, et al.** Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from Lactobacillus pentosus with a fungistatic effect on *Candida albicans*. *J Appl Microbiol* (1999); 87(5):726–34.

[191] **Goffeng AR, Holst E, Nilsson C, et al.** Microorganisms in vaginal fluid from women in prolonged pregnancy.

Gynecol Obstet Invest (1997); 44(1):16–20.

[192] **Goffeng AR, Holst E, Milsom I, et al.** Fetal fibronectin and microorganisms in vaginal fluid of healthy pregnant women. *Acta Obstet Gynecol Scand* (1996); 75(6):520–5.

[193] **Xu J, Sobel JD**. Antibiotic-associated vulvovaginal candidiasis. *Curr Infect Dis Rep* (2003); 5(6):481–7.

[194] **Ness RB, Hillier SL, Richter HE, et al.** Why women douche and why they may or may not stop. *Sex Transm Dis* (2003); 30(1):71–4.

[195] **Tarry W, Fisher M, Shen S, et al.** *Candida albicans*: the estrogen target for vaginal colonization. *J Surg Res* (2005); 129(2):278–82.

[196] **Boskey ER**. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Hum Reprod* (2001); 16(9):1809–13.

[197] **De Bernardis F, Muhlschlegel FA, Cassone A, et al.** The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect Immun* (1998); 66(7):3317–25.

[198] **Muhlschlegel F, Fonzi W**. PHR2 of *Candida albicans* encodes a functional homolog of the pH- regulated gene PHR1 with an inverted pattern of pH-dependent expression. *Mol Cell Biol* (1997); 17(10):5960–7.

[199] **Ferreira C, Gonçalves B, Vilas Boas D, et al.** *Candida tropicalis* biofilm and human epithelium invasion is highly influenced by environmental pH. *Pathog Dis* (2016); 74(8):ftw101.

[200] **Hilton E**. Ingestion of yogurt containing Lactobacillus acidophilus as prophylaxis for candidal vaginitis. *Ann Intern Med* (1992); 116(5):353–7.

[201] **Hawes SE, Hillier SL, Benedetti J, et al.** Hydrogen peroxide-producing lactobacilli and acquisition of vaginal Infections. *J Infect Dis* (1996); 174(5):1058–63.

[202] **Rathod SD, Klausner JD, Krupp K, et al.** Epidemiologic features of vulvovaginal candidiasis among reproductive-age women in India. *Infect Dis Obstet Gynecol* (2012); 2012:1–8.

[203] **Spinillo A, Carratta L, Pizzoli G, et al.** Recurrent vaginal candidiasis. Results of a cohort study of sexual transmission and intestinal reservoir. *J Reprod Med* (1992); 37(4):343–7.

[204] **Jeavons HS**. Prevention and treatment of vulvovaginal candidiasis using exogenous Lactobacillus. *J Obstet Gynecol Neonatal Nurs* (2003); 32(3):287–96.

[205] **De Seta F, Parazzini F, De Leo R, et al.** Lactobacillus plantarum P17630 for preventing *Candida* vaginitis recurrence: a retrospective comparative study. *Eur J Obstet Gynecol Reprod Biol* (2014); 182:136–9.

[206] **Murina F, Graziottin A, Vicariotto F, et al.** Can Lactobacillus fermentum LF10 and Lactobacillus acidophilus LA02 in a slow-release vaginal product be useful for prevention of recurrent vulvovaginal candidiasis?: A clinical study. *J Clin Gastroenterol* (2014); 48 Suppl 1:S102-5.

[207] **Richardson M, Rautemaa R**. How the host fights against *Candida* infections. *Front Biosci (Landmark Ed* (2009); 14:4363–75.

[208] **Duerr A, Heilig CM, Meikle SF, et al.** Incident and persistent vulvovaginal candidiasis among human immunodeficiency virus–infected women: Risk factors and severity. *Obstet Gynecol* (2003); 101(3):548–56.

[209] **Zitvogel L, Apetoh L, Ghiringhelli F, et al.** Immunological aspects of cancer chemotherapy. *Nat Rev Immunol* (2008); 8(1):59–73.

[210] **Fidel PL, Sobel JD**. Immunopathogenesis of recurrent vulvovaginal candidiasis. *Clin Microbiol Rev* (1996); 9(3):335–48.

[211] **Witkin SS**. Immunologic factors influencing susceptibility to recurrent candidal vaginitis. *Clin Obstet Gynecol* (1991); 34(3):662–8.

[212] **Mostad SB, Overbaugh J, DeVange DM, et al.** Hormonal contraception, vitamin A deficiency, and other risk factors for shedding of HIV-1 infected cells from the cervix and vagina. *Lancet* (1997); 350(9082):922–7.

[213] **Schuman P, Sobel JD, Ohmit SE, et al.** Mucosal candidal colonization and candidiasis in women with or at risk for Human Immunodeficiency Virus infection. *Clin Infect Dis* (1998); 27(5):1161–7.

[214] **Apalata T, Longo-Mbenza B, Sturm A, et al.** Factors associated with symptomatic vulvovaginal candidiasis: a

study among women attending a primary healthcare clinic in Kwazulu-Natal, South Africa. *Ann Med Health Sci Res* (2014); 4(3):410–6.

[215] **Schuman P, Capps L, Peng G, et al.** Weekly fluconazole for the prevention of mucosal candidiasis in women with HIV infection. A randomized, double-blind, placebo-controlled trial. Terry Beirn Community Programs for Clinical Research on AIDS. *Ann Intern Med* (1997); 126(9):689–96.

[216] **Wira CR, Fahey J V, Sentman CL, et al.** Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol Rev* (2005); 206:306–35.

[217] **Medzhitov R**. Toll-like receptors and innate immunity. *Nat Rev Immunol* (2001); 1(2):135–45.

[218] **Brown GD, Gordon S**. Immune recognition of fungal beta-glucans. *Cell Microbiol* (2005); 7(4):471–9.

[219] **Jouault T, El Abed-El Behi M, Martinez-Esparza M, et al.** Specific recognition of *Candida albicans* by macrophages requires galectin-3 to discriminate Saccharomyces cerevisiae and needs association with TLR2 for signaling. *J Immunol* (2006); 177(7):4679–87.

[220] **Netea MG, Brown GD, Kullberg BJ, et al.** An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat Rev Microbiol* (2008); 6(1):67–78.

[221] **Ip W-K, Lau Y-L**. Role of mannose-binding lectin in the innate defense against *Candida albicans*: enhancement of complement activation, but lack of opsonic function, in phagocytosis by human dendritic cells. *J Infect Dis* (2004); 190(3):632–40.

[222] **Pellis V, De Seta F, Crovella S, et al.** Mannose binding lectin and C3 act as recognition molecules for infectious agents in the vagina. *Clin Exp Immunol* (2005); 139(1):120–6.

[223] **Ferwerda B, Ferwerda G, Plantinga TS, et al.** Human dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med* (2009); 361(18):1760–7.

[224] **Babula O, Lazdane G, Kroica J, et al.** Relation between recurrent vulvovaginal candidiasis, vaginal concentrations of mannose-binding lectin, and a mannose-binding lectin gene polymorphism in Latvian women. *Clin Infect Dis* (2003); 37(5):733–7.

[225] **Giraldo PC, Babula O, Gonçalves AKS, et al.** Mannose-binding lectin gene polymorphism, vulvovaginal candidiasis, and bacterial vaginosis. *Obstet Gynecol* (2007); 109(5):1123–8.

[226] **Fidel PL, Luo W, Steele C, et al.** Analysis of vaginal cell populations during experimental vaginal candidiasis. *Infect Immun* (1999); 67(6):3135–40.

[227] **Fulurija A, Ashman RB, Papadimitriou JM**. Neutrophil depletion increases susceptibility to systemic and vaginal candidiasis in mice, and reveals differences between brain and kidney in mechanisms of host resistance. *Microbiology* (1996); 142(12):3487–96.

[228] **Liu Y-J**. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* (2001); 106(3):259–62.

[229] **Rosentul D, Delsing C, Joosten LAB, et al.** Polymorphism in innate immunity genes and susceptibility to recurrent vulvovaginal candidiasis. *J Mycol Médicale / J Med Mycol* (2009); 19(3):191–6.

[230] **Babovic-Vuksanovic D, Snow K, Ten RM**. Mannose-binding lectin (MBL) deficiency. Variant alleles in a midwestern population of the United States. *Ann Allergy Asthma Immunol* (1999); 82(2):134–8.

[231] **Barousse MM, Espinosa T, Dunlap K, et al.** Vaginal epithelial cell anti-*Candida albicans* activity is associated with protection against symptomatic vaginal candidiasis. *Infect Immun* (2005); 73(11):7765–7.

[232] **Wira CR, Grant-Tschudy KS, Crane-Godreau MA**. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. *Am J Reprod Immunol* (2005); 53(2):65–76.

[233] **Barnhart KT**. Baseline dimensions of the human vagina. *Hum Reprod* (2006); 21(6):1618–22.

[234] **Farage M, Maibach H**. Lifetime changes in the vulva and vagina. *Arch Gynecol Obstet* (2005); 273(4):195–202.

[235] **Cremel M**. Characterization of CCL20 secretion by human epithelial vaginal cells: involvement in Langerhans cell precursor attraction. *J Leukoc Biol* (2005); 78(1):158–66.

[236] **King AE, Critchley HO, Kelly RW**. Presence of secretory leukocyte protease inhibitor in human endometrium

and first trimester decidua suggests an antibacterial protective role. *Mol Hum Reprod* (2000); 6(2):191–6.

[237] **Quayle AJ, Porter EM, Nussbaum AA, et al.** Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol* (1998); 152(5):1247–58.

[238] **Ganz T**. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* (2003); 3(9):710–20.

[239] **Wira CR, Fahey J V, Ghosh M, et al.** Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *Am J Reprod Immunol* (2010); 63(6):544–65.

[240] **Barousse MM, Steele C, Dunlap K, et al.** Growth inhibition of *Candida albicans* by human vaginal epithelial cells. *J Infect Dis* (2001); 184(11):1489–93.

[241] **Talaei Z, Sheikhbahaei S, Ostadi V, et al.** Recurrent vulvovaginal candidiasis: could it be related to cell-mediated immunity defect in response to *Candida* antigen? *Int J Fertil Steril* (2017); 11(3):134–41.

[242] **Cassone A, De Bernardis F, Santoni G**. Anticandidal immunity and vaginitis: novel opportunities for immune intervention. *Infect Immun* (2007); 75(10):4675–86.

[243] **Pudney J, Quayle AJ, Anderson DJ**. Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone. *Biol Reprod* (2005); 73(6):1253–63.

[244] **Fidel PL**. Immunity in vaginal candidiasis. *Curr Opin Infect Dis* (2005); 18(2):107–11.

[245] **Anjuère F, Bekri S, Bihl F, et al.** B cell and T cell immunity in the female genital tract: Potential of distinct mucosal routes of vaccination and role of tissue-associated dendritic cells and natural killer cells. *Clin Microbiol Infect* (2012); 18:117–22.

[246] **Rodriguez Garcia M, Patel M V., Shen Z, et al.** Mucosal immunity in the human female reproductive tract. *Mucosal Immunol* (2015):2097–124.

[247] **Carvalho LP, Bacellar O, Neves N, et al.** Downregulation of IFN-γ production in patients with recurrent vaginal candidiasis. *J Allergy Clin Immunol* (2002); 109(1):102–5.

[248] **Kalo-Klein A, Witkin SS**. Prostaglandin E2 enhances and gamma interferon inhibits germ tube formation in *Candida albicans*. *Infect Immun* (1990); 58(1):260–2.

[249] **Fidel, PL J, Lynch M, Sobel J**. Circulating CD4 and CD8 T cells have little impact on host defense against experimental vaginal candidiasis. *Infect Immun* (1995); 63(7):2403–8.

[250] **Cenci E, Romani L, Mencacci A, et al.** Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of *Candida albicans*. *Eur J Immunol* (1993); 23(5):1034–8.

[251] **Pietrella D, Rachini A, Pines M, et al.** Th17 cells and IL-17 in protective immunity to vaginal candidiasis. *PLoS One* (2011); 6(7):e22770.

[252] **Carvalho RJV de, Cunha CM, Silva DA de O, et al.** IgA, IgE e subclasses de IgG anti-*Candida albicans* no soro e lavado vaginal de pacientes com candidíase vulvovaginal. *Rev Assoc Med Bras* (2003); 49(4):434–8.

[253] **Wira CR, Veronese F**. Mucosal immunity in the male and female reproductive tract and prevention of HIV transmission. *Am J Reprod Immunol* (2011); 65(3):182–5.

[254] **Silva CRG e, Melo KE de, Leão MVP, et al.** Presença de *Candida* nas mucosas vaginal e bucal e sua relação com IgA salivar. *Rev Bras Ginecol e Obs* (2008); 30(6):300–5.

[255] **Fidel PL, Ginsburg KA, Cutright JL, et al.** Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: evidence for vaginal Th1-type responses following intravaginal challenge with *Candida* antigen. *J Infect Dis* (1997); 176(3):728–39.

[256] **Gough PM, Warnock DW, Richardson MD, et al.** IgA and IgG antibodies to *Candida albicans* in the genital tract secretions of women with or without vaginal candidosis. *Med Mycol* (1984); 22(4):265–71.

[257] **Russell MW, Mestecky J**. Humoral immune responses to microbial infections in the genital tract. *Microbes Infect* (2002); 4(6):667–77.

[258] **Fan SR, Liao QP, Liu XP, et al.** Vaginal allergic response in women with vulvovaginal candidiasis. *Int J Gynaecol*

Obstet (2008); 101(1):27–30.

[259] **Regúlez P, García Fernández JF, Moragues MD, et al.** Detection of anti-*Candida albicans* IgE antibodies in vaginal washes from patients with acute vulvovaginal candidiasis. *Gynecol Obstet Invest* (1994); 37(2):110–4.

[260] **Yildirim Z, Kilic N, Kalkanci A**. Fluorometric determination of acid proteinase activity in *Candida albicans* strains from diabetic patients with vulvovaginal candidiasis. *Mycoses* (2011); 54(5):e463–7.

[261] **Bohannon NJ V.** Treatment of vulvovaginal candidiasis in patients with diabetes. *Diabetes Care* (1998); 21(3):451–6.

[262] **Gunther LSA, Martins HPR, Gimenes F, et al.** Prevalence of *Candida albicans* and non-*albicans* isolates from vaginal secretions: comparative evaluation of colonization, vaginal candidiasis and recurrent vaginal candidiasis in diabetic and non-diabetic women. *Sao Paulo Med J* (2014); 132(2):116–20.

[263] **Atabek ME, Akyürek N, Eklioglu BS**. Frequency of vagınal *Candida* colonization and relationship between metabolic parameters in children with type 1 diabetes mellitus. *J Pediatr Adolesc Gynecol* (2013); 26(5):257–60.

[264] **Banerjee K, Curtis E, San Lazaro C, et al.** Low prevalence of genital candidiasis in children. *Eur J Clin Microbiol Infect Dis* (2004); 23(9):696–8.

[265] **Peer AK, Hoosen AA, Seedat MA, et al.** Vaginal yeast infections in diabetic women. *S Afr Med J* (1993); 83(10):727–9.

[266] **Ray D, Goswami R, Banerjee U, et al.** Prevalence of *Candida* and its response to boric acid vaginal suppositories in comparison with oral fluconazole in patients with diabetes and vulvovaginal candidiasis. *Diabetes Care* (2007); 30(2):312–7.

[267] **de Leon EM, Jacober SJ, Sobel JD, et al.** Prevalence and risk factors for vaginal *Candida* colonization in women with type 1 and type 2 diabetes. *BMC Infect Dis* (2002); 2:1.

[268] **Masharani U, German M**. Pancreatic hormones and diabetes mellitus. *Greenspan's Basic Clin. Endocrinol.* (2011).

[269] **Donders GGG, Prenen H, Verbeke G, et al.** Impaired tolerance for glucose in women with recurrent vaginal candidiasis. *Am J Obstet Gynecol* (2002); 187(4):989–93.

[270] **Rodrigues CF, Rodrigues ME, Henriques M**. *Candida sp.* infections in patients with diabetes mellitus. *J Clin Med* (2019); 8(1):76.

[271] **Mikamo H, Yamagishi Y, Sugiyama H, et al.** High glucose-mediated overexpression of ICAM-1 in human vaginal epithelial cells increases adhesion of *Candida albicans*. *J Obstet Gynaecol (Lahore)* (2018); 38(2):226–30.

[272] **Pereira IDB, Souza AES de, Feio RS, et al.** Vulvovaginites por *Candida albicans* em pacientes ambulatoriais do Hospital Universitário Betina Ferro de Souza. *Rev bras anal clin* (1996); 28(2):53–4.

[273] **Hostetter MK**. Handicaps to host defense. Effects of hyperglycemia on C3 and *Candida albicans*. *Diabetes* (1990); 39(3):271–5.

[274] **de Souza Ferreira C, Araújo TH, Ângelo ML, et al.** Neutrophil dysfunction induced by hyperglycemia: modulation of myeloperoxidase activity. *Cell Biochem Funct* (2012); 30(7):604–10.

[275] **Nash EE, Peters BM, Lilly EA, et al.** A murine model of *Candida glabrata* vaginitis shows no evidence of an inflammatory immunopathogenic response. *PLoS One* (2016); 11(1):e0147969.

[276] **Ferris HA, Kahn CR**. New mechanisms of glucocorticoid-induced insulin resistance: make no bones about it. *J Clin Invest* (2012); 122(11):3854–7.

[277] **Barnes PJ**. Glucocorticosteroids: current and future directions. *Br J Pharmacol* (2011); 163(1):29–43.

[278] **Fukushima C, Matsuse H, Tomari S, et al.** Oral candidiasis associated with inhaled corticosteroid use: comparison of fluticasone and beclomethasone. *Ann Allergy Asthma Immunol* (2003); 90(6):646–51.

[279] **Bradford LL, Ravel J, Bruno V**. Understanding vulvovaginal candidiasis through a community genomics approach. *Curr Fungal Infect Rep* (2013); 7(2):126–31.

[280] **Antonio MAD, Hawes SE, Hillier SL**. The identification of vaginal Lactobacillus species and the demographic and microbiologic characteristics of women colonized by these species. *J Infect Dis* (1999); 180(6):1950–6.

[281] **Chaim W, Foxman B, Sobel JD**. Association of recurrent vaginal candidiasis and secretory ABO and Lewis phenotype. *J Infect Dis* (1997); 176(3):828–30.

[282] **Hilton E, Chandrasekaran V, Rindos P, et al.** Association of recurrent candidal vaginitis with inheritance of Lewis blood group antigens. *J Infect Dis* (1995); 172(6):1616–9.

[283] **Gow NAR, Netea MG, Munro CA, et al.** Immune recognition of *Candida albicans* beta-glucan by dectin-1. *J Infect Dis* (2007); 196(10):1565–71.

[284] **Brown GD**. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol* (2006); 6(1):33–43.

[285] **Plantinga TS, van der Velden WJFM, Ferwerda B, et al.** Early stop polymorphism in human Dectin-1 is associated with increased *Candida* colonization in hematopoietic stem cell transplant recipients. *Clin Infect Dis* (2009); 49(5):724–32.

[286] **Neth O, Jack DL, Dodds AW, et al.** Mannose-binding lectin binds to a range of clinically relevant microorganisms and promotes complement deposition. *Infect Immun* (2000); 68(2):688–93.

[287] **Liu F, Liao Q, Liu Z**. Mannose-binding lectin and vulvovaginal candidiasis. *Int J Gynaecol Obstet* (2006); 92(1):43–7.

[288] **Burchard EG, Silverman EK, Rosenwasser LJ, et al.** Association between a sequence variant in the IL-4 gene promoter and FEV 1 in asthma. *Am J Respir Crit Care Med* (1999); 160(3):919–22.

[289] **Babula O, Lazdane G, Kroica J, et al.** Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. *Clin Infect Dis* (2005); 40(9):1258–62.

[290] **Lev-Sagie A, Prus D, Linhares IM, et al.** Polymorphism in a gene coding for the inflammasome component NALP3 and recurrent vulvovaginal candidiasis in women with vulvar vestibulitis syndrome. *Am J Obstet Gynecol* (2009); 200(3):303.e1-6.

[291] **Egbe CA, Onwufor UC, Omoregie R, et al.** Female reproductive tract infections among vaginal contraceptive users in Benin city, Nigeria. *Genomic Med Biomarkers, Heal Sci* (2011); 3(1):49–52.

[292] **Oviasogie F, Okungbowa F**. *Candida* species amongst pregnant women In Benin City, Nigeria: effect of predisposing factors. *African J Clin Exp Microbiol* (2009); 10(2):92–8.

[293] **Reed BD, Gorenflo DW, Gillespie BW, et al.** Sexual behaviors and other risk factors for *Candida* vulvovaginitis. *J Womens Health Gend Based Med* (2000); 9(6):645–55.

[294] **Sobel JD, Wiesenfeld HC, Martens M, et al.** Maintenance fluconazole therapy for recurrent vulvovaginal candidiasis. *N Engl J Med* (2004); 351(9):876–83.

[295] **Rylander E, Berglund A-L, Krassny C, et al.** Vulvovaginal *Candida* in a young sexually active population: prevalence and association with oro-genital sex and frequent pain at intercourse. *Sex Transm Infect* (2004); 80(1):54–7.

[296] **Spinillo A, Pizzoli G, Colonna L, et al.** Epidemiologic characteristics of women with idiopathic recurrent vulvovaginal candidiasis. *Obstet Gynecol* (1993); 81(5 (Pt 1)):721–7.

[297] **Fleetwood L, Landgren B-M, Eneroth P**. Changes in soluble proteins in cervical mucus during midcycle in normally menstruating women. *Gynecol Obstet Invest* (1984); 18(1):27–33.

[298] **Abasiattai A, Bassey E, Udoma E**. Profile of intrauterine contraceptive device acceptors at the university of Uyo teaching hospital, Uyo, Nigeria. *Ann Afr Med* (2008); 7(1):1.

[299] **Pruthi V, Al-Janabi A, Pereira BMJ**. Characterization of biofilm formed on intrauterine devices. *Indian J Med Microbiol* (2003); 21(3):161–5.

[300] **Nguyen Y, Fischer G**. Chronic vulvovaginal candidiasis in patients using a levonorgestrel-containing intrauterine device. *Australas J Dermatol* (2018); 59(1):e39–42.

[301] **Agarwal K, Sharma U, Acharya V**. Microbial and cytopathological study of intrauterine contraceptive device users. *Indian J Med Sci* (2004); 58(9):394–9.

[302] **Auler ME, Morreira D, Rodrigues FFO, et al.** Biofilm formation on intrauterine devices in patients with recurrent vulvovaginal candidiasis. *Med Mycol* (2010); 48(1):211–6.

[303] **Zahran KM, Agban MN, Ahmed SH, et al.** Patterns of *Candida* biofilm on intrauterine devices. *J Med Microbiol* (2015); 64(Pt_4):375–81.

[304] **Pascual LM, Daniele MB, Pájaro C, et al.** Lactobacillus species isolated from the vagina: identification, hydrogen peroxide production and nonoxynol-9 resistance. *Contraception* (2006); 73(1):78–81.

[305] **Wilkinson D, Tholandi M, Ramjee G, et al.** Nonoxynol-9 spermicide for prevention of vaginally acquired HIV and other sexually transmitted infections: systematic review and meta-analysis of randomised controlled trials including more than 5000 women. *Lancet Infect Dis* (2002); 2(10):613–7.

[306] **Watts DH, Rabe L, Krohn MA, et al.** The effects of three nonoxynol-9 preparations on vaginal flora and epithelium. *J Infect Dis* (1999); 180(2):426–37.

[307] **Roddy RE, Cordero M, Cordero C, et al.** A dosing study of nonoxynol-9 and genital irritation. *Int J STD AIDS* (1993); 4(3):165–70.

[308] **Stafford MK, Ward H, Flanagan A, et al.** Safety study of nonoxynol-9 as a vaginal microbicide: evidence of adverse effects. *J Acquir Immune Defic Syndr Hum Retrovirol* (1998); 17(4):327–31.

[309] **McGroarty JA, Soboh F, Bruce AW, et al.** The spermicidal compound nonoxynol-9 increases adhesion of *Candida* species to human epithelial cells in vitro. *Infect Immun* (1990); 58(6):2005–7.

[310] **Giraldo PC, Amaral RLG do, Gonçalves AK, et al.** Influência da freqüência de coitos vaginais e da prática de duchas higiênicas sobre o equilíbrio da microbiota vaginal. *Rev Bras Ginecol e Obs* (2005); 27(5):257–62.

[311] **Schreiber CA, Meyn LA, Creinin MD, et al.** Effects of long-term use of nonoxynol-9 on vaginal flora. *Obstet Gynecol* (2006); 107(1):136–43.

[312] **Foxman B**. The epidemiology of vulvovaginal candidiasis: risk factors. *Am J Public Health* (1990); 80(3):329–31.

[313] **Hellberg D, Zdolsek B, Nilsson S, et al.** Sexual behavior of women with repeated episodes of vulvovaginal candidiasis. *Eur J Epidemiol* (1995):575–9.

[314] **Markos AR, Wade AA, Walzman M**. Oral sex and recurrent vulvo-vaginal candidiasis. *Genitourin Med* (1992); 68(1):61–2.

[315] **Reed BD, Zazove P, Pierson CL, et al.** *Candida* transmission and sexual behaviors as risks for a repeat episode of *Candida* vulvovaginitis. *J Women's Heal* (2003); 12(10):979–89.

[316] **Zeng X, Zhang Y, Zhang T, et al.** Risk factors of vulvovaginal candidiasis among women of reproductive age in Xi'an: a cross-sectional study. *Biomed Res Int* (2018); 2018:1–8.

[317] **Holanda AAR de, Fernandes ACS, Bezerra CM, et al.** Candidíase vulvovaginal: sintomatologia, fatores de risco e colonização anal concomitante. *Rev Bras Ginecol e Obs* (2007); 29(1):3–9.

[318] **Ekuma AE, Ikenyi CL, Moses AE**. *Candida* colonization and genital hygiene habits among women in Uyo, Nigeria. *J Res BASIC Clin Sci* (2019); 1(1):17–22.

[319] **Neves NA, Carvalho LP, De Oliveira MAM, et al.** Association between atopy and recurrent vaginal candidiasis. *Clin Exp Immunol* (2005); 142(1):167–71.

[320] **Arfiputri DS, Hidayati AN, Handayani S, et al.** Risk factors of vulvovaginal candidiasis in dermato-venereology outpatients clinic of Soetomo general hospital, Surabaya, Indonesia. *African J Infect Dis* (2018); 12(1 Suppl):90–4.

[321] **Watson C, Calabretto H**. Comprehensive review of conventional and non-conventional methods of management of recurrent vulvovaginal candidiasis. *Aust New Zeal J Obstet Gynaecol* (2007); 47(4):262–72.

[322] **Faraji R, Rahimi M, Assarehzadegan M**. Prevalence of vaginal candidiasis infection in women referred to Kermanshah hygienic centers, Iran in 2010. *Life Sci J* (2012); 9(4):1280–3.

Chapter 2

Biofilm formation of *Candida* vaginal isolates at acidic versus neutral pH

Main goal

To study the influence of pH (4 and 7) on virulence factors (filamentation, biofilm formation and biofilm matrix composition) of vaginal isolates of the two most common species in vulvovaginal candidiasis, *Candida albicans* and *Candida glabrata*.

Conclusions

This study showed that *C. albicans* and *C. glabrata* are able to growth and display relevant virulence features in either acidic or neutral conditions, presenting a response to pH dependent on species and strain, what makes them challenging pathogens in the vaginal environment.

This chapter is based on the following article:

Gonçalves B, Fernandes L, Henriques M, Silva S. Biofilm formation of *Candida* vaginal isolates at acidic versus neutral pH (submitted).

2.1 Introduction

The vaginal environment is acidic, being a pH between 3.6 and 4.5 considered normal for women with active menstrual cycles [1]. In those women, the hormone stimulated vaginal mucosa produces glycogen that is deposited in vaginal epithelial cells [2]. When cells need energy, glycogen is converted to glucose, which is metabolized anaerobically within the vaginal mucosa leading to the production of lactic acid as a byproduct [3]. Additionally, *Lactobacillus* species, that predominate in the vaginal environment and whose proliferation is potentiated at acidic conditions, also metabolize extracellular glycogen into lactic acid by anaerobic glycolysis [1,3]. As such, it has been suggested that a complex biological system is operative in the vagina, leading to the vaginal acidity through the production of lactic acid by the vaginal epithelium and bacteria [3].

A close relation between ovarian hormones and vaginal pH is demonstrated by an increased vaginal pH in women with low hormone levels, as in-prepuberty and post-menopause, in which the pH can range from 5.5 to 8.0 [1,2]. Other factors, not related with hormonal function, may also lead to temporary increase of vaginal pH, including semen [4], vaginal douching [1], blood and medications [2,5], but the relative contributions of these factors are unclear. Additionally, in absence of recent exposure to semen, menstruation or exogenous products, an elevated pH (>4.5) in healthy reproductive-age women is suggestive of bacterial vaginosis (BV) or a sexually transmitted infection as *Chlamydia trachomatis, Neisseria gonorrhoeae* or *Trichomonas vaginalis* [2,6]**.** Importantly, although the vaginal acidity is considered an important barrier against the majority of vaginal infections, *Candida* species can infect the vulvovaginal tract at normal acidic pH. Thus, the presence of symptoms of vaginal infection with normal vaginal pH strongly suggests vulvovaginal candidiasis (VVC) [3]. Nevertheless, *Candida* species can also provoke a concomitant infection with BV, which occurs at higher vaginal pH [7], demonstrating high ability of vaginal species to adapt to different environmental pH. In fact, *Candida* species are opportunistic fungi that can colonize and potentially infect host niches with pH that may range from 2 (stomach) to 8 (gut), what makes them important and challenging pathogens [8].

Environmental pH has significant effects on *Candida* cells, mainly at the plasma membrane, including on protein activity, proton gradient and nutrient availability [9]. A detailed study on the influence of pH on *Candida tropicalis* growth revealed significant differences in its biofilm formation ability and invasion of reconstituted human vaginal epithelium at acidic and neutral conditions [10]**.** Additionally, pH has been shown to be an important signal for morphological differentiation in *Candida albicans* [9,11]. As such, this study aimed the analysis of growth and virulence factors of vaginal isolates of the two most

common *Candida* species in VVC, *C. albicans* and *Candida glabrata*, at acidic conditions potentiated by lactic acid, and their comparison with neutral conditions.

2.2 Materials and Methods

2.2.1 *Candida* strains and initial culture conditions

In this study one reference strain and two vaginal isolates of *C. albicans* (SC5314, 557843 and 569322, respectively) and *C. glabrata* (ATCC 2001, 534784, 585626, respectively) were used. The isolates belong to the Biofilm Group collection (Centre of Biological Engineering, University of Minho, Braga, Portugal) and were isolated from patients of Hospital of S Marcos, Braga, Portugal. The identity of the isolates was confirmed using CHROMagar *Candida* (CHROMagar, Paris, France) and PCR-based identification with specific primers (ITS1 and ITS4) [12].

Candida strains were grown on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) at 37 °C for 48 h. For each experiment, some colonies of SDA plates were used to inoculate 40 ml of Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany), incubated for 18 h at 37 °C under agitation (120 rev/min). The cellular suspensions were then centrifuged at 5000 g for 10 min at 4 °C and the cells washed twice with 10 ml of ultra-pure sterile water. The supernatants were discarded and pellets suspended in Roswell Park Memorial Institute medium (RPMI; Sigma-Aldrich, St Louis, MO, USA), buffered with 3-(N-Morpholino) propanesulfonic acid (MOPS; Sigma-Aldrich, St Louis, MO, USA) and adjusted to pH 4.0 with lactic acid or to pH 7.0 with sodium hydroxide. For the subsequent planktonic growth or biofilm development, the cellular density was adjusted to $1x10^5$ cells/ml, using a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany). The experiments described in next sections were performed in triplicate (same pre-inoculum) and in three independent assays (pre-inoculums independently prepared).

2.2.2 Analysis of planktonic growth

For the planktonic growth, the cellular suspensions of *C. albicans* and *C. glabrata* strains prepared in RPMI at pH 4.0 and 7.0 were placed in 25 ml Erlenmeyer flasks and incubated for 24 h at 37°C under

agitation in an orbital shaker (120 rev/min). The increase in optical density at 690 nm was measured over time using a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, VT, USA).

Additionally, *C. albicans* strains were also evaluated in terms of filamentous forms formation. For that, after the 24 h of incubation, aliquots of each cellular suspension were diluted in sterile water (1:100) and filamentous forms were counted in an optical microscope using a Neubauer chamber (results were presented as percentage of filamentous forms).

2.2.3 Biofilm formation

In order to develop *C. albicans* and *C. glabrata* biofilms, the cellular suspensions, prepared in RPMI at pH 4.0 and 7.0 were placed into wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) (200 µl per well) and incubated for 24 h at 37°C under agitation (120 rev/min) [13]. After incubation, the medium was aspirated and biofilms were washed with 200 µl of Phosphate Buffered Saline (PBS) to remove non-adherent cells. The biofilms were analysed as described in the next sections.

2.2.4 Biofilm biomass quantification

The biofilms' total biomass was analysed by Crystal Violet (CV) staining methodology [13]. For that, previously washed biofilms were firstly fixed with methanol (200 µl per well) for 15 min. After methanol removal the microtiter plates were dried at room temperature and then, 200 µl of CV (1% v/v) were added to each well. The stain was aspirated after 5 min and its excess was removed by washing the biofilms twice with sterile water. Finally, 200 µl of acetic acid (33%, v/v) were added to each well and the solutions were homogenized to release and dissolve the stain. The absorbance of the solutions was measured in a microtiter plate reader at 570 nm and the results were presented as absorbance per unit area (Absorbance CV/cm^2).

2.2.5 Determination of biofilm cells' cultivability

The number of cultivable cells on biofilms was measured by colony forming units (CFU) counting methodology [14]. Briefly, washed biofilms were scraped from the microtiter plates wells with 200 µl of PBS per well and the suspensions were vigorously vortexed for 2 min to disaggregate the cells from matrix

[14]. Then, a serial 10-fold dilutions were prepared in PBS with the planktonic suspensions, which were plated on SDA and incubated for 24 h at 37 °C. After incubation, the number of CFU on SDA plates was counted and the results were presented per unit area (Log (CFU/ml)/cm^2).

2.2.6 Biofilm structure and thickness analysis

Confocal laser scanning microscope (CLSM) was used to analyse biofilm structure and thickness. For that, biofilms were developed for 24 h as previously described, but 24-well polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) were used instead (1 ml of suspension per well). Biofilms were stained with 1% (v/v) of Calcofluor white (Sigma-Aldrich, St Louis, MO, USA) for 10 min at room temperature in the dark and then observed with a CLSM (Olympus BX61, Model FluoView 1000, Portugal). The excitation line 405 and the emission filters BA 430-470 (blue channel) were used, and images were acquired with the program FV10-ASW 4.2 (Olympus, Portugal).

2.2.7 Analysis of biofilm cells' filamentous forms

In order to quantify the number of filamentous forms in *C. albicans* biofilm cells, biofilms were scraped from the microtiter plate's wells with PBS and the suspensions vortexed for 2 min to disaggregate the cells [14]. Then, aliquots of these suspensions were used to determine the percentage of filamentous forms as described above for planktonic cells. The filamentous forms of biofilm cells were also visualized by CLSM, as described in the previous section.

2.2.8 Biofilm matrix extraction and analysis

In order to study the effect of pH on biofilm matrix, biofilms of *C. albicans* and *C. glabrata* were developed as described above, using 24-well polystyrene microtiter plates. Developed biofilms were scraped from the wells, resuspended in ultra-pure water and sonicated (Ultrasonic Processor, Cole-Parmer, Vernon Hills, IL, USA) for 30 s at 30 W, in order to separate cells from biofilm matrix [13]. Then, the suspensions were centrifuged at 5000 g for 5 min at 4°C. The pellets were dried at 37°C until a constant weight was obtained [15]. The matrix-containing supernatants were filtered through a 0.2 μm nitrocellulose filter and their contents of protein, total carbohydrate and (1,3)-β-D-glucan were quantified.

2.2.8.1 Protein quantification and analysis

The protein content was measured using the BCA Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, MO, USA) and bovine serum albumin (BSA) as the standard [13]. Briefly, 0.2 ml of BCA solution were added to 25 µl of each sample and incubated for 30 min at 37°C. Then, the absorbance was determined in a microplate reader at 562 nm. Protein concentration was extrapolated from a calibration curve (abs = 0.009 [protein] +0.1685) previously performed using standard concentrations of BSA. The results were normalized with the biofilm cells' dry weight determined and presented as mg of protein per g of biofilm dry weight (mg/$g_{biofilm}$).

The matrix of *C. glabrata* ATCC 2001 biofilm was also analysed by nano Liquid chromatography-tandem mass spectrometry (LC-MS/MS) in order to identify the proteins secreted in each condition. First the protein samples were digested following an already described procedure [16] with some modifications. Briefly, protein extracts were diluted in an 8 M urea/100 mM Triethylamonium bicarbonate (TEAB) solution, centrifuged and washed in the same solution. Next, protein samples were reduced and alkylated with 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM Carboxylic acid amide (CAA), in 8 M urea/100 mM TEAB for 30 min in the dark at 30°C, centrifuged, followed by a first wash in 8 M urea/100 mM TEAB and a second wash in 50 mM TEAB. Protein digestion with trypsin/Lys-C mix was performed overnight at 37°C in 50 mM TEAB. The reaction was stopped with 1% Trifluoroacetic acid (TFA). Peptides were then recovered by centrifugation followed by an additional centrifugation step with 0.1% TFA. Next, peptide samples were cleaned-up and concentrated by chromatography. Protein identification was then performed by nano LC-MS/MS, in an equipment composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) [17]. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.8 software (Thermo Scientific, Bremen, Germany). The raw data were processed using Proteome Discoverer 2.2.0.388 software (Thermo Scientific) and searched against the UniProt database for the taxonomic selection *C. glabrata*. The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragment ions. Maximum allowed missing cleavage sites was set to 2 and peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target FDR 1%, validation of based on q-value.

2.2.8.2 Carbohydrate quantification

Total carbohydrate content was estimated with the phenol-sulfuric method [18], using glucose as standard. Briefly, 0.5 ml of phenol (50 g/l) and 2.5 ml of sulfuric acid (95-97%) were added to 0.5 ml of each matrix sample. This solution was vortexed for 30 s and incubated for 15 min at room temperature. The absorbance was read in a microtiter plate reader at 490 nm. The concentration of the carbohydrate was extrapolated from a calibration curve (Abs = 0.2955[carbohydrate] + 0.114) previously performed with standard glucose concentrations. The results were normalized with the biofilm cells' dry weight and presented as mg of carbohydrate per g of biofilm dry weight ($mg/g_{biofilm}$).

2.2.8.3 (1,3)-β-D-glucan quantification

The concentration of (1,3)-β-D-glucan was determined using the commercially available Glucatell (1,3)-β-D-Glucan Detection Reagent kit (Associates of Cape Cod Inc, East Falmouth, MA, USA). Briefly, 50 µl of Glucatell reagent were added to 50 µl of each matrix sample and incubated at 37°C for 30 min. The reaction was stopped by adding 50 µl of sodium nitrite and in sequence 50 µl of ammonium sulfamate and 50 µl of N-(1-Napthyl) ethylenediamine dihydrochloride. The optical density was read at 540 nm and the concentration of (1,3)-β-D-glucan was extrapolated from a calibration curve performed with standard glucan concentrations (Abs = 0.0106[glucans] + 0.0392). The results were normalized with the biofilm cells' dry weight and presented as ng of (1,3)-β-D-glucan per g of biofilm dry weight ($ng/g_{biofilm}$).

2.2.9 Statistical analysis

Results of biofilm biomass, cells cultivability, filamentation and matrix components' quantities were statistically analysed using GraphPad Prism 6 software. For that, the results obtained at pH 4.0 and 7.0 were compared, for each strain, using the t test analysis. All tests were performed with a confidence level of 95%.

2.3 Results

2.3.1 *Candida albicans* and *C. glabrata* planktonic growth at acidic and neutral conditions

In order to analyse the influence of pH on planktonic growth of *C. albicans* and *C. glabrata* strains, the optical density of free-floating cells cultivated in RPMI buffered to pH 4 and 7 was accompanied for 24 h. The results (Figure 2.1) show that all *C. albicans* and *C. glabrata* strains had the expected growth stages, at both pH values tested. The planktonic growth was similar at pH 4 and 7, although slightly slower at neutral conditions in *C. albicans* strains. Additionally, the growth of the reference strain and vaginal isolates was similar, in both species and conditions (Figure 2.1).

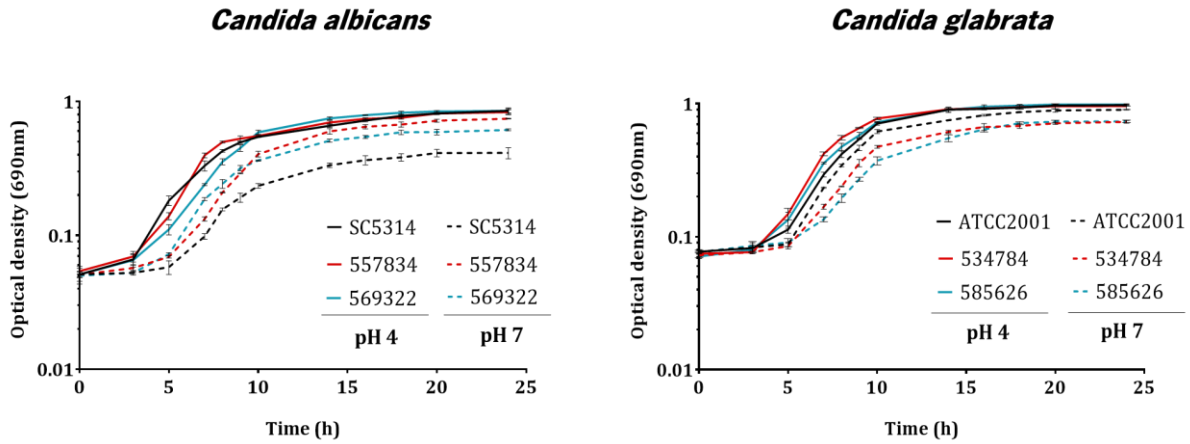


Figure 2.1 *Candida albicans* and *Candida glabrata* planktonic growth at pH 4 and pH 7. Growth curves (optical density, of *C. albicans* SC5314 (reference), 557834 and 569322 (vaginal isolates) and *C. glabrata* ATCC 2001 (reference), 537834 and 585626 (vaginal isolates), performed over 24 h of growth at pH 4 and 7. Error bars represent standard deviation.

2.3.2 *Candida albicans* and *C. glabrata* biofilm formation at acidic and neutral conditions

In order to study the influence of pH on the biofilm formation ability of *C. albicans* and *C. glabrata* strains, biofilms were developed for 24 h in RPMI at pH 4 and 7, and analysed in terms of total biomass (Figure 2.2-A) and cultivable cells (Figure 2.2-B). It was evident that all *C. albicans* and *C. glabrata* strains

tested have the ability to form biofilms at both pH, but the influence of pH on biofilm formation was different in each species.

Candida albicans strains presented statistically higher (P-value ≤0.05) biofilm biomass at neutral conditions, comparatively to the acidic environment, with an average increase of 20%, in the reference strain (SC5314) and vaginal isolates (557834, 569322) (Figure 2.2-A). Consistently, a higher number of cultivable cells was found in *C. albicans* biofilms developed at pH 7 than pH 4, although the results were not statistically different (P-value >0.05) (Figure 2.2-B). In contrast, *C. glabrata* biofilms developed at pH 7 presented statistically lower biomass (P-value ≤0.01) and number of cultivable cells (P-value ≤0.05) than those developed at pH 4. *Candida glabrata* biofilms presented a decrease of 24 % in average, on biomass (Figure 2.2-A), and of approximately 1 order of magnitude, on cultivable cells (Log (CFU/ml)/cm²) (Figure 2.2-B), in the reference strain (ATCC 2001) and vaginal isolates (534784, 585626).

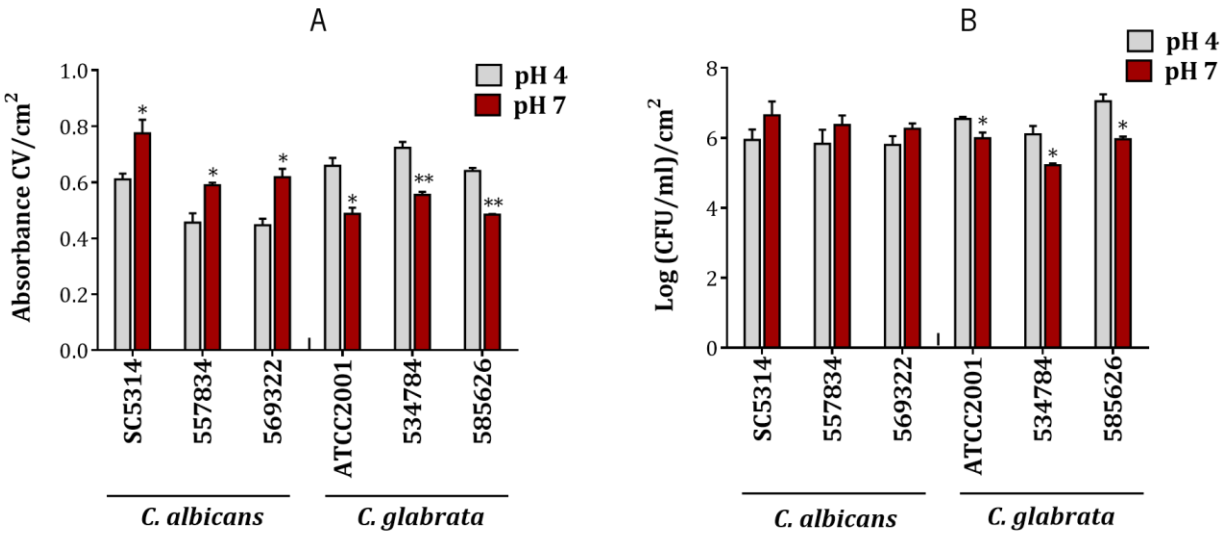


Figure 2.2 *Candida albicans* and *Candida glabrata* biofilm formation at pH 4 and pH 7. (A) Total biomass quantification (Absorbance CV/cm²) and **(B)** cultivable cells determination (Log (CFU/ml)/cm²) of *C. albicans* SC5314 (reference), 557834 and 569322 (vaginal isolates) and *C. glabrata* ATCC 2001 (reference), 537834 and 585626 (vaginal isolates) biofilms, developed for 24 h at pH 4 and pH 7. Error bars represent standard deviation. Asterisks represent statistical difference between the results obtained at pH 4 and pH 7 (** P-value ≤0.01; * P-value ≤0.05).

Candida albicans and *C. glabrata* biofilms were further visualized by CLSM in order to analyse the influence of pH on their structure and thickness. The obtained images (Figures 2.3 and 2.4), show that *C. albicans* and *C. glabrata* biofilms presented alterations in their thickness and structure depending on the pH used.

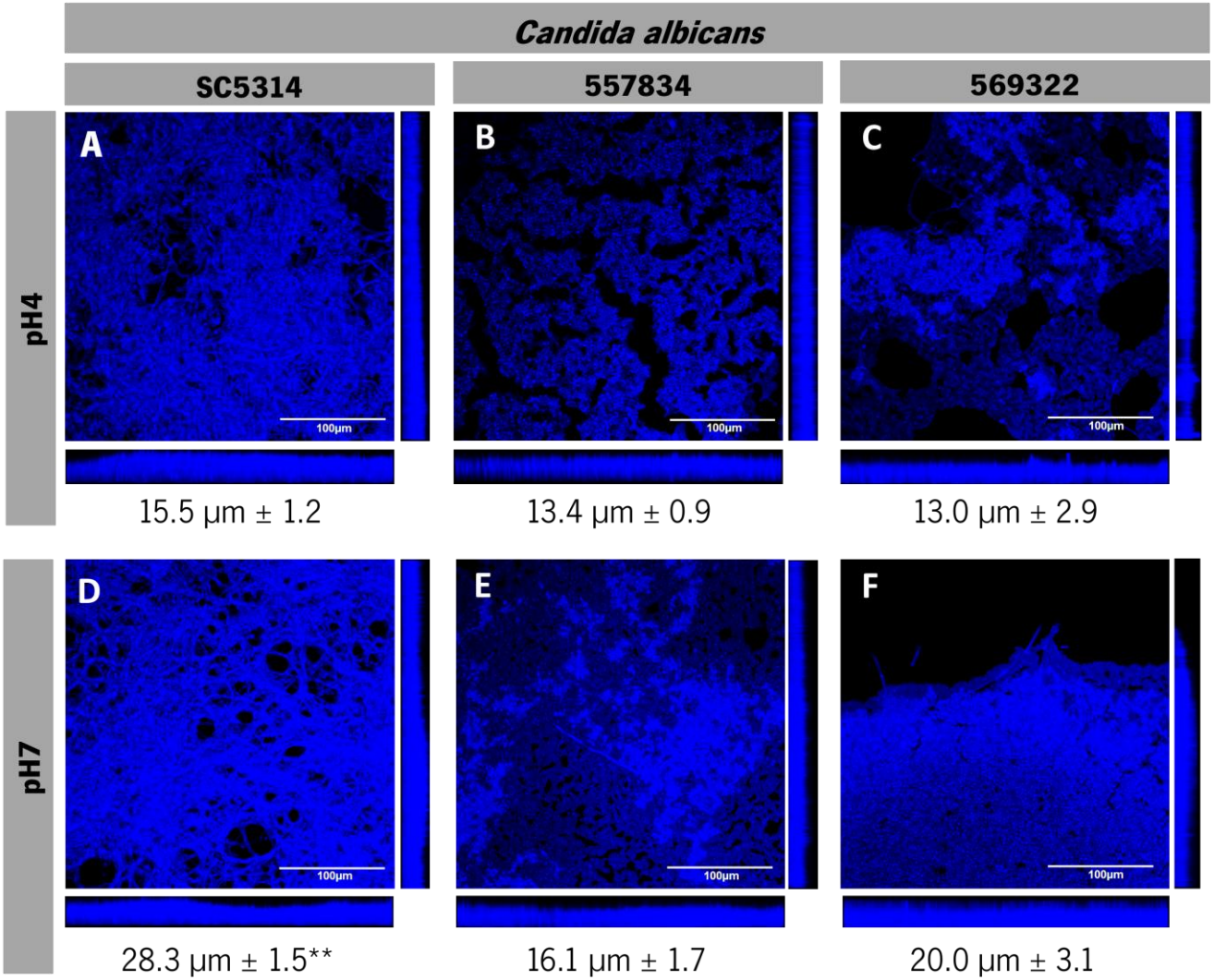


Figure 2.3 Effect of pH on *Candida albicans* biofilm structure and thickness. Confocal laser scanning microscopy images of *C. albicans* SC5314 (A,D), 557834 (B,E) and 569322 (C,F) biofilms, developed for 24 h at pH 4 and pH 7. The values of thickness are presented as means ± standard deviations. Asterisks represent statistical difference in the results obtained at pH 7 compared to pH 4 (** P-value ≤0.01). Original magnification was 40x and the scale bars correspond to 100 µm.

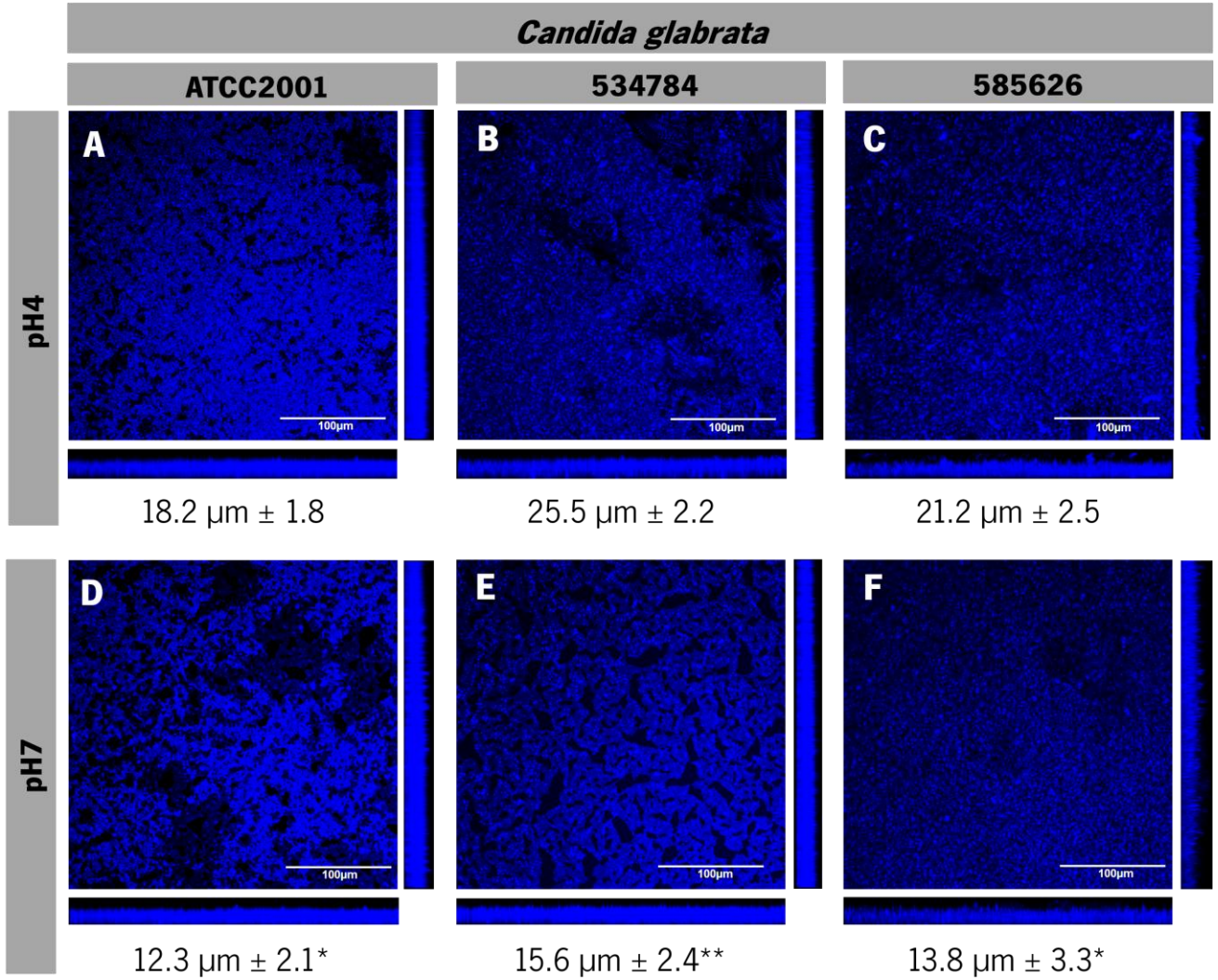


Figure 2.4 Effect of pH on *Candida glabrata* biofilm structure and thickness. Confocal laser scanning microscopy images of *C. glabrata* ATCC 2001 (A,B), 537834 (B,E) and 585626 (C,F) biofilms, developed for 24 h at pH 4 and pH 7. The values of thickness are presented as means ± standard deviations. Asterisks represent statistical difference in the results obtained at pH 7 compared to pH 4 (** P-value ≤0.01; *P-value ≤0.05). Original magnification was 40x and the scale bars correspond to 100 µm.

In *C. albicans* strains, higher biofilm thickness was detected at pH 7 compared to pH 4 (Figure 2.3), consistent with the results obtained in the previous analyses. Biofilms of the reference strain (*C. albicans* SC5314) presented a statistically higher thickness at pH 7 (P-value ≤0.01) and a clear difference in their structure, being observed an intense network of filamentous forms at neutral conditions (Figure 2.3-D). In the vaginal isolates, structural differences were also observed between the conditions, and the biofilms of all *C. albicans* strains formed at pH 7 seem to have a more glistening appearance than at pH 4 (Figure 2.3).

In contrast, and in accordance with the previous analyses, all *C. glabrata* biofilms presented statistically lower (P-value ≤0.05) biofilm thickness at pH 7 compared to pH 4 (Figure 2.4). Biofilm structural differences were also observed in all strains, especially in *C. glabrata* ATC2001 and 534784, being the biofilm of the reference strain apparently more cohesive at pH 7 (Figure 2.4-A).

2.3.3 Influence of pH on *C. albicans* filamentation

Candida albicans strains grown in planktonic and biofilm lifestyles at pH 4 and 7 were analysed by optical microscopy in order to evaluate the influence of pH on their filamentous forms' development (Figure 2.5).

All strains presented higher percentage of cells in the filamentous form after growing at pH 7 than at pH 4, independently of the growth lifestyle (Figure 2.5-A). *Candida albicans* SC5314 and 585626 presented statistically higher (P-value ≤0.05) percentage of cells in the filamentous form at pH 7, corresponding, approximately, to additional 50 and 10% of cells with filaments, respectively, in both lifestyles (Figure 2.5-A). Although *C. albicans* 557834 presented low ability to produce filaments in planktonic or biofilm lifestyle, an increase was also observed at pH 7 compared to pH 4.

The filamentous forms' length present in biofilms developed at pH 4 and 7 was also evaluated by CLSM by selecting a conveying zone of the biofilm in which the cells in the filamentous form are visible at high resolution (Figure 2.5-B). In the reference strain it was clear that the filaments length is much higher at pH 7. *Candida albicans* 569322 had a similar behavior, but the filaments at pH 4 seem to have a longer length than those of *C. albicans* SC5314 in the same condition. In *C. albicans* 557834 no filaments were found at pH 4 and those at pH 7 seem to have lower length than in other strains and were harder to be visualized (Figure 2.5-B).

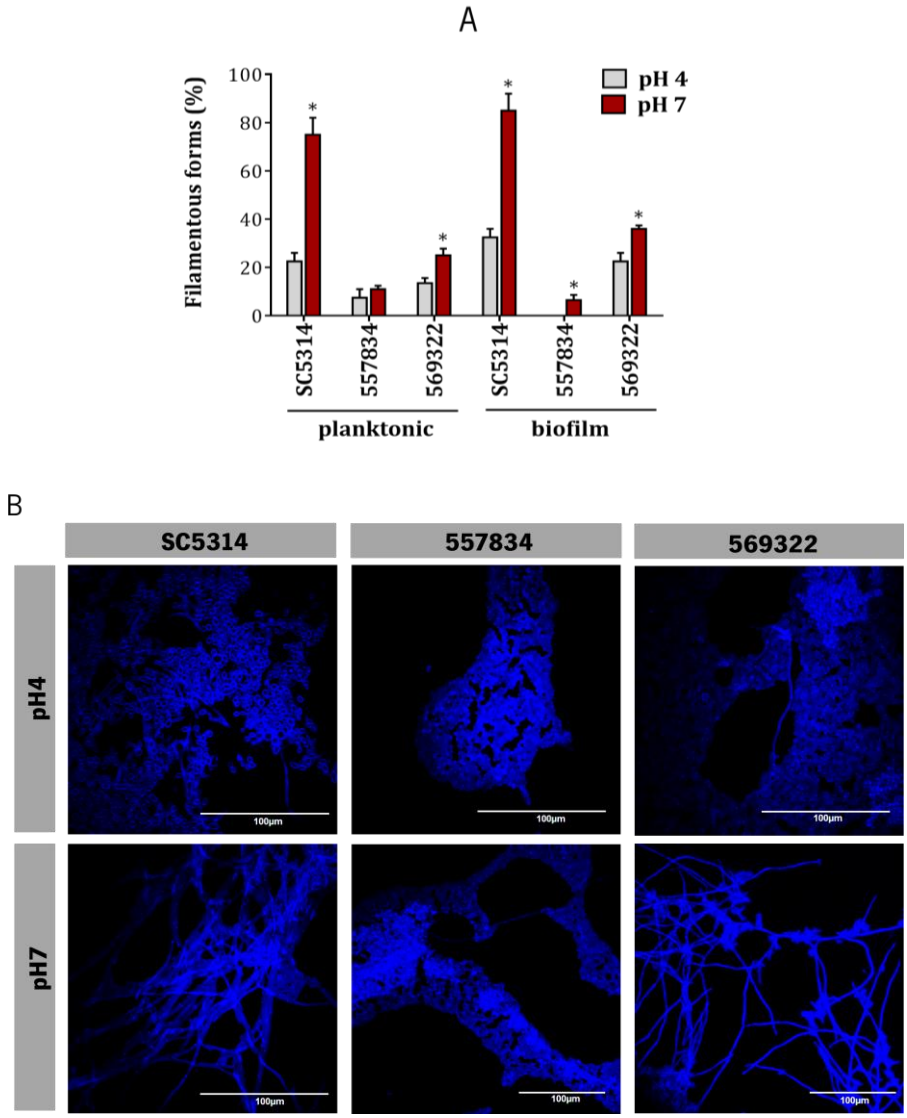


Figure 2.5 Effect of pH on *Candida albicans* filamentous forms development. (A) Filamentous forms percentage of *C. albicans* SC5314, 557834 and 569322 cells grown in planktonic and biofilm lifestyles for 24 h at pH 4 and pH 7. **(B)** Confocal laser scanning microscopy images of *C. albicans* SC5314, 557834 and 569322 biofilm cells grown at pH 4 and 7. Error bars represent standard deviation. Asterisks represent statistical differences between the results obtained at pH 4 and pH 7 (* P-value ≤0.05). Original magnification was 60x and the scale bars correspond to 100 µm.

2.3.4 Influence of pH on *C. albicans* and *C. glabrata* biofilm matrix

The matrices of *C. albicans* and *C. glabrata* biofilms developed at pH 4 and 7 were also analysed in order to evaluate the influence of pH on the production of their major components, namely protein and carbohydrate (total content and (1,3)-β-D-glucan). The results (Table 2.1) show that pH has a significant influence on the amount of *C. albicans* and *C. glabrata* biofilm matrix components, that was shown to be dependent on species and strain.

Table 2.1 Influence of pH on *Candida albicans* and *Candida glabrata* biofilm matrix production. Quantification of protein, carbohydrate and (1,3)-β-D-glucan in the matrix of *C. albicans* SC5314, 557834, 569322 and *C. glabrata* ATCC 2001, 537834 and 585626 biofilms developed for 24 h at pH 4 and pH 7. The results were normalized with the respective biofilm dry weight and presented as means ± standard deviations. Asterisks represent statistical differences between the results obtained at pH 4 and pH 7 (****P-value ≤0.0001; *** P-value ≤0.001; ** P-value ≤0.01; * P-value ≤0.05).

Species	Strain	pH	Protein (mg/g biofilm)	Carbohydrate (mg/g biofilm)	(1,3)-β-D-glucan (ng/g biofilm)
Candida albicans	SC5314	**4**	42.92 ± 5.11	148.48 ± 11.37	47.47 ± 1.43
		7	64.31 ± 11.39	578.20 ± 10.26***	73.65 ± 6.57*
	557834	**4**	56.39 ± 7.27	398.48 ± 13.16	50.40 ± 2.94
		7	65.00 ± 6.09	889.20 ± 12.79***	75.47 ± 3.40*
	569322	**4**	50.69 ± 7.07	123.94 ± 5.38	54.00 ± 2.84
		7	77.36 ± 6.68	839.93 ± 7.25****	78.34 ± 5.70*
Candida glabrata	ATCC 2001	**4**	37.68 ± 8.64	104.23 ± 13.4	45.91 ± 1.16
		7	73.89 ± 4.52 *	511.42 ± 6.58***	83.82 ± 2.94**
	534784	**4**	57.92 ± 1.18	99.83 ± 11.96	62.79 ± 6.62
		7	59.74 ± 6.1	772.42 ± 6.78***	89.75 ± 2.28*
	585626	**4**	55.28 ± 6.87	296.39 ± 5.18	55.73 ± 3.35
		7	29.31 ± 7.46	167.94 ± 16.15**	41.02 ± 0.42*

All *C. albicans* strains showed increased production of matrix components in biofilms formed at pH 7 compared to pH 4, however the impact of pH had some differences among the strains tested (Table 2.1). The total carbohydrate content was statistically higher at neutral conditions, in all strains (P-value ≤0.01), being the highest increase presented by *C. albicans* 569322, of about six times, followed by reference strain that presented an amount almost four times higher (Table 2.1). Consistently, all *C. albicans* strains presented statistically higher (P-value ≤0.05) amount of (1,3)-β-D-glucan in the matrices of biofilms formed at neutral than acidic conditions (Table 2.1). The increase of (1,3)-β-D-glucan was about 1.5 times in all strains. Additionally, the protein content was also higher in the biofilm matrices of all strains at neutral conditions, although the results were not statistically different from the acidic environment (P-value >0.05).

Among *C. glabrata* strains there were some differences in the influence of pH in the production of matrix components. The amount of carbohydrate and protein contents found in *C. glabrata* 585626 biofilm matrix at pH 7 was almost half of that obtained at pH 4 (Table 2.1). In contrast, the biofilms of the reference strain and the vaginal isolate *C. glabrata* 534784 formed at pH 7, presented higher amount of matrix components than those developed at pH 4. The biofilms of these strains developed at neutral conditions presented statistically higher (P-value ≤0.001) amount of carbohydrate in their matrices, of about five and seven times the amount found at pH 4, respectively (Table 2.1). Consistently, these biofilms presented statistically higher (P-value ≤0.05) amount of (1,3)-β-D-glucan and also higher quantity of protein in their matrices when developed at neutral conditions (Table 2.1).

The biofilms of *C. glabrata* ATCC 2001 presented the most relevant and the only statistically significant difference (P-value ≤0.05) in the matrix protein content between the two pH values (Table 2.1). As such, the proteins present in the matrix of *C. glabrata* ATCC 2001 biofilms developed in both conditions were also identified in order determine the influence of pH on *C. glabrata* biofilm secretome profile. The results (Figure 2.6) show that 326 proteins were commonly present in both conditions, 71 proteins were only present at pH 4 and 280 only at pH 7, or at least were the only proteins present in a level that allowed their detection by the methodology used.

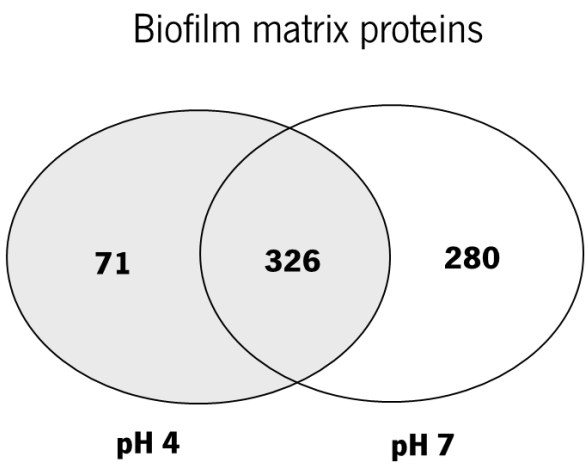


Figure 2.6 Influence of pH on *Candida glabrata* biofilm matrix proteins. Venn diagram showing the number of proteins found in the matrix of *C. glabrata* ATCC 2001 biofilms developed for 24 h at pH 4 and/or pH 7.

2.4 Discussion

The vaginal acidity, promoted by the production of lactic acid by bacteria and vaginal epithelium, is considered important for a healthy vaginal environment in pre-menopause women as it confers a biological barrier against most vaginal infections [2]. However, *Candida* species colonize and usually infect the vulvovaginal tract at normal acidic pH [3]. Additionally, they can also provoke VVC at elevated vaginal pH [7]. As such, it was aimed to study the growth and some virulence factors of *C. albicans* and *C. glabrata* vaginal isolates using culture media at pH 7 and pH 4, the latter achieved with lactic acid to mimic the vaginal acidity.

The evaluation of planktonic growth (Figure 2.1) revealed that all the strains tested (one reference and two vaginal isolates of each species) have the ability to grow at pH 4 and pH 7. *Candida albicans* strains were found to grow slower at pH 7 than pH 4 (Figure 2.1), in accordance with previous studies reporting higher *C. albicans* growth rate at acidic than neutral or alkaline environments [11,19]. These studies also report lower filamentation ability at acidic conditions, that was also observed in this study (Figure 2.5-A). In contrast, *C. glabrata* strains appear to grow similarly at acidic and neutral conditions (Figure 2.1), in accordance with a previous report [20]. So, it may be suggested that the observed effects of pH on *C. albicans* and *C. glabrata* planktonic cells are independent of strain or other environmental factors, as different strains and conditions were used for those studies and ours. Nevertheless, the level of impact of pH is probably influenced by environmental factors, as it presented differences among the studies.

Interestingly, *C. albicans* strains presented significantly higher amount of total biofilm biomass (Figure 2.2-A) and thickness (Figure 2.3) at pH 7 compared to pH 4, being the highest quantity presented by the reference strain, at both pH values. Additionally, the pH was also found to affect the biofilm structure of all strains (Figure 2.3). The reference strain presented an intense filamentation at pH 7, not seen at pH 4, corroborated by the finding of higher percentage of biofilm cells in the filamentous form (Figure 2.5-A) and increased filaments length (Figure 2.5-B). These results are in accordance with a previous study that also found higher *C. albicans* SC5314 biofilm thickness and filamentation at neutral than acidic conditions (pH 5.6) [21]. The pH had a similar influence on the biofilms of the vaginal isolates, although the filamentation ability of these strains was different from the reference strain. *Candida albicans* 557834 presented low ability to filament and *C. albicans* 534784 lower percentage of filaments but with higher size than the reference strain at pH 4 (Figure 2.5). The differences presented by the strains in their filamentation or biofilm structure show that *C. albicans* strains can present significant differences among common virulence factors, what may influence their pathogenic potential. Additionally, the higher

filamentation ability found at neutral conditions may enhance *C. albicans* tissue invasion capability [22], but importantly, it has been suggested that an acidic environment lowers *C. albicans* cells ability to filament in order to induce biofilm dispersion, leading to new foci of infection [23].

In contrast, all *C. glabrata* strains presented lower ability to produce biofilms at neutral conditions compared to the acidic environment (Figure 2.2), presenting biofilms with different structures at different pH conditions (Figure 2.4). A previous study did not found significant influence of pH on *C. glabrata* development (pH 7 compared to pH 5.6) [21]. However, Schmidt et al. [20] studied *C. glabrata* proteome and found that this species perceives low pH as less stressful than higher pH, what may possibly promote its biofilm formation at pH 4. These authors also reported that the high ability of *C. glabrata* to adapt to acidic conditions is consistent with the idea that *C. glabrata* is an opportunistic pathogen of humans that had evolved primarily in relatively acidic environmental niches such as root fruit [20]. It is important to address that the acid used in this study was different from those used in previous studies, and may have a specific effect on *C. glabrata* biofilm development. The high ability of *C. glabrata* species to develop biofilms at an acidic environment with lactic acid may contribute to the high difficulty to treat VVC provoked by this species [24,25]. Additionally, this ability may be related with the common isolation of *C. glabrata* strains in women with recurrent VVC, that is potentially related with biofilms presence [26,27].

One of the most important characteristics of *Candida* biofilms is the extracellular matrix, which functions are not entirely clear but it is thought to provide architectural strength, to control the biofilm desegregation and to protect against antifungal agents and the host immune system [28,29]. The relative production of matrix contents revealed higher content of carbohydrate than protein, in all strains and conditions. These results are in accordance with some studies [13,29,30] and in contrast with others (only *C. albicans*) [31,32]. These differences potentially reflect the different strains and experimental setups used between the studies.

The neutral pH was found to increase the production of matrix components in all *C. albicans* biofilms (Table 2.1), consistent with the glistening appearance of the biofilms observed at pH 7 (Figure 2.3), a feature that has been proposed to be associated with the accumulation of extracellular matrix [33]. Total carbohydrate was the matrix component showing the highest increase, but the pH impact was strain dependent. *Candida albicans* 534784 presented the highest increase and *C. albicans* 557834 the highest carbohydrate amount in the biofilm matrix among *C. albicans* strains (Table 2.1). To the author's knowledge this is the first study on the influence of pH on *Candida* biofilm matrix production and composition. Importantly, it has been demonstrated a predominant role for matrix carbohydrate, especially of mannan and glucan components, in the resistance of *C. albicans* to antifungal agents,

through a mechanism of drug sequestration that difficult the drug from reaching their cellular targets [31,34]. As such, higher content of matrix carbohydrate at neutral conditions may reflect higher biofilm resistance to antifungals, in accordance with a previous study that reported higher *C. albicans* biofilm resistance to caspofungin at neutral than acidic conditions [21]. Caspofungin exert its activity by inhibiting (1,3)-β-D-glucan synthases [35] and in fact we found that (1,3)-β-D-glucan content in *C. albicans* biofilms matrix is increased by neutral pH, although the results suggest that other carbohydrate components may be more affected by the environmental pH (Table 2.1). Indeed, it was recently shown [32] that, contrarily to what was previously thought, (1,3)-β-D-glucan, which represents the major cell wall carbohydrate and was previously demonstrated to play a major role in biofilm drug resistance [34,36], only comprise a small portion of the total carbohydrate fraction of the biofilm matrix, being mannans the main component [32]. Interestingly a study of the influence of pH on planktonic cells found lower *C. albicans* susceptibility, to several antifungal agents, at acidic compared to neutral conditions, suggesting that the increase of biofilm antifungal resistance by neutral conditions is specific for this growth mode [37].

In *C. glabrata* strains the influence of pH on biofilm matrix composition was strain dependent. The reference strain and one vaginal isolate (*C. glabrata* 534784) presented a response to pH similar to that of *C. albicans*, with increased quantity of matrix components, mainly carbohydrate, in biofilms developed at neutral conditions (Table 2.1). It should be noted that, although these strains possess higher amount of matrix components at neutral conditions in a normalized quantity of biofilm, it was observed that their ability to produce biofilm was higher at acidic conditions and thus the total amount of matrix could potentially be higher at pH 4. In accordance, higher resistance to caspofungin and anidulafungin was reported for *C. glabrata* ATCC 2001 biofilms at acidic than neutral conditions [21]. In contrast with other strains *C. glabrata* 585626 presented higher production of biofilm matrix components at pH 4 (Table 2.1). Consistently, *C. glabrata* biofilms presented structural differences between the conditions and also between strains (Figure 2.4). These findings may reflect inherent physiological differences between *C. glabrata* strains that could have significance with respect to their response to drugs and host defences.

Candida glabrata ATCC 2001 was the only strain that presented a significant change of matrix protein quantity between the conditions tested (Table 2.1). So, its biofilms' secretome was analysed and it was revealed that, besides influencing the quantity of protein secreted, the environmental pH had a significant influence on the identity of proteins secreted to the matrix, being found a high number specifically secreted at pH 4 and 7 (Figure 2.6). Interestingly, neutral conditions not only increase the production of matrix proteins as also increases the number of specific proteins secreted. It should be noted that undetected proteins may not be totally absent but were at least at an undetectable level in

matrix samples with similar concentration. Contrary to carbohydrates the function of matrix proteins and their identity is largely unknown, especially in *C. glabrata*. In *C. albicans* a recent study reported, for the first time, the proteomic profile of its biofilm matrix, using a technique similar to that used in this study, and found 565 different proteins, proposing functions as nutrition and biofilm cells dispersion [32]. This number was pretty close to the number of proteins found in this study for *C. glabrata* at neutral conditions (606 proteins). At pH 4 a lower number of total proteins (397) were found but a significant number (71 proteins) were specifically secreted at pH 4. These results suggest a specific profile and regulation of *C. glabrata* biofilm matrix proteins at acidic conditions that are analysed in detail in Chapter 3.

Adaptation to changes in pH during biofilm formation is crucial not only for its development, but also for survival of microbial communities at colonized mucosa or medical devices (as intrauterine devices). Different pH response between species and strains is clinically relevant as increase the difficulty to manage VVC prevention and treatment.

References

[1] **García-Closas M, Herrero R, Bratti C, et al.** Epidemiologic determinants of vaginal pH. *Am J Obstet Gynecol* (1999); 180(5):1060–6.

[2] **Caillouette JC, Sharp CF, Zimmerman GJ, et al.** Vaginal pH as a marker for bacterial pathogens and menopausal status. *Am J Obstet Gynecol* (1997); 176(6):1270–7.

[3] **Linhares IM, Summers PR, Larsen B, et al.** Contemporary perspectives on vaginal pH and lactobacilli. *Am J Obstet Gynecol* (2011); 204(2):120.e1-120.e5.

[4] **Bouvet J-P, Grésenguet G, Bélec L**. Vaginal pH neutralization by semen as a cofactor of HIV transmission. *Clin Microbiol Infect* (1997); 3(1):19–23.

[5] **Polatti F, Rampino M, Magnani P, et al.** Vaginal pH-lowering effect of locally applied vitamin C in subjects with high vaginal pH. *Gynecol Endocrinol* (2006); 22(4):230–4.

[6] **Donders G, Van Calsteren K, Bellen G, et al.** Predictive value for preterm birth of abnormal vaginal flora, bacterial vaginosis and aerobic vaginitis during the first trimester of pregnancy. *BJOG An Int J Obstet Gynaecol* (2009); 116(10):1315–24.

[7] **Fidel PL, Vazquez JA, Sobel JD**. *Candida glabrata* : review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin Microbiol Rev* (1999); 12(1):80–96.

[8] **Vylkova S, Carman AJ, Danhof HA, et al.** The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH. *MBio* (2011); 2(3):e00055-11.

[9] **Davis D**. Adaptation to environmental pH in *Candida albicans* and its relation to pathogenesis. *Curr Genet* (2003); 44(1):1–7.

[10] **Ferreira C, Gonçalves B, Vilas Boas D, et al.** *Candida tropicalis* biofilm and human epithelium invasion is highly influenced by environmental pH. *Pathog Dis* (2016); 74(8):ftw101.

[11] **Konno N, Ishii M, Nagai A, et al.** Mechanism of *Candida albicans* transformation in response to changes of pH. *Biol Pharm Bull* (2006); 29(5):923–6.

[12] **Williams DW, Wilson MJ, Lewis MAO, et al.** Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* (1995); 33(9):2476–9.

[13] **Silva S, Henriques M, Martins A, et al.** Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* (2009); 47(7):681–9.

[14] **Silva S, Henriques M, Oliveira R, et al.** *In vitro* biofilm activity of non-*Candida albicans Candida* species. *Curr Microbiol* (2010); 61(6):534–40.

[15] **Rodrigues CF, Gonçalves B, Rodrigues ME, et al.** The effectiveness of voriconazole in therapy of *Candida glabrata*'s biofilms oral infections and its influence on the matrix composition and gene expression. *Mycopathologia* (2017).

[16] **Wiśniewski JR, Zougman A, Nagaraj N, et al.** Universal sample preparation method for proteome analysis. *Nat Methods* (2009); 6(5):359–62.

[17] **Michalski A, Damoc E, Hauschild J-P, et al.** Mass spectrometry-based proteomics using Q exactive, a high-performance benchtop Quadrupole Orbitrap mass spectrometer. *Mol Cell Proteomics* (2011); 10(9):M111.011015.

[18] **DuBois M, Gilles KA, Hamilton JK, et al.** Colorimetric method for determination of sugars and related substances. *Anal Chem* (1956); 28(3):350–6.

[19] **Davis D, Wilson RB, Mitchell AP**. RIM101-dependent and-independent pathways govern pH responses in *Candida albicans*. *Mol Cell Biol* (2000); 20(3):971–8.

[20] **Schmidt P, Walker J, Selway L, et al.** Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*. *Proteomics* (2008); 8(3):534–44.

[21] **Kucharikova S, Tournu H, Lagrou K, et al.** Detailed comparison of *Candida albicans* and *Candida glabrata* biofilms under different conditions and their susceptibility to caspofungin and anidulafungin. *J Med Microbiol* (2011); 60(9):1261–9.

[22] **Gow NA., Brown AJ., Odds FC**. Fungal morphogenesis and host invasion. *Curr Opin Microbiol* (2002); 5(4):366–71.

[23] **Uppuluri P, Chaturvedi AK, Srinivasan A, et al.** Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog* (2010); 6(3):e1000828.

[24] **Sojakova M, Liptajova D, Borovsky M, et al.** Fluconazole and itraconazole susceptibility of vaginal yeast isolates from Slovakia. *Mycopathologia* (2004); 157(2):163–9.

[25] **Richter SS, Galask RP, Messer SA, et al.** Antifungal susceptibilities of Candida species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol* (2005); 43(5):2155–62.

[26] **Amouri I, Sellami H, Borji N, et al.** Epidemiological survey of vulvovaginal candidosis in Sfax, Tunisia. *Mycoses* (2011); 54(5):e499-505.

[27] **Grigoriou O, Baka S, Makrakis E, et al.** Prevalence of clinical vaginal candidiasis in a university hospital and possible risk factors. *Eur J Obstet Gynecol Reprod Biol* (2006); 126(1):121–5.

[28] **Donlan RM, Costerton JW**. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* (2002); 15(2):167–93.

[29] **Al-Fattani MA, Douglas LJ**. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* (2006); 55(Pt 8):999–1008.

[30] **Baillie GS, Douglas LJ**. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* (2000); 46(3):397–403.

[31] **Dominguez E, Zarnowski R, Sanchez H, et al.** Conservation and divergence in the *Candida* species biofilm matrix mannan-glucan complex structure, function, and genetic control. *MBio* (2018); 9(2).

[32] **Zarnowski R, Westler WM, Lacmbouh GA, et al.** Novel entries in a fungal biofilm matrix encyclopedia. *MBio* (2014); 5(4):e01333-14.

[33] **Nobile CJ, Nett JE, Hernday AD, et al.** Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol* (2009); 7(6):e1000133.

[34] **Nett JE, Crawford K, Marchillo K, et al.** Role of Fks1p and matrix glucan in *Candida albicans* biofilm resistance to an echinocandin, pyrimidine, and polyene. *Antimicrob Agents Chemother* (2010); 54(8):3505–8.

[35] **Andriole VT**. Current and future antifungal therapy: new targets for antifungal therapy. *Int J Antimicrob Agents* (2000); 16(3):317–21.

[36] **Nett J, Lincoln L, Marchillo K, et al.** Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob Agents Chemother* (2007); 51(2):510–20.

[37] **Danby CS, Boikov D, Rautemaa-Richardson R, et al.** Effect of pH on in vitro susceptibility of *Candida glabrata* and *Candida albicans* to 11 antifungal agents and implications for clinical use. *Antimicrob Agents Chemother* (2012); 56(3):1403–6.

Chapter 3

Biofilm matrix regulation by *Candida glabrata* Zap1 under acidic conditions

Main goal

To study the regulation of biofilm formation and matrix composition by the *Candida glabrata*'s transcriptional factor Zap1, at acidic conditions.

Conclusions

This study showed that Zap1 is a negative regulator of *C. glabrata* biofilm matrix production, affecting the quantity of carbohydrates and proteins. Additionally, for the first time, this study revealed the *C. glabrata* biofilm matrix proteomic profile and its complex regulation by Zap1.

This chapter is based on the following article:

Gonçalves B, Pires DP, Azevedo NM, Fernandes L, Henriques M, Silva S. Biofilm matrix proteome regulation by *Candida glabrata* Zap1under acidic conditions (submitted).

3.1 Introduction

Candida glabrata is a clinically relevant pathogen of the vaginal tract, that has been commonly isolated in patients with recurrent vulvovaginal candidiasis (RVVC), which has a potential relation to biofilms, as they may hamper the complete eradication of the fungus [1,2]. Biofilms have unique phenotypic characteristics compared to their planktonic counterpart cells, as the presence of an extracellular matrix, which derives from directed synthesis and secretion of components, as well as, lysis of a fraction of biofilm cells [3]. Although the matrix role is not fully understood, it has been suggested to mediate adhesive and cohesive interactions, providing a mechanically stable infrastructure for biofilm accumulation and controlling biofilm dispersion [4]. Additionally, the biofilm matrix has been shown to play an important role in the protection of biofilms from harmful environments as those promoted by the host immune system or in presence of antifungal drugs [4]. Importantly, matrix composition and regulation have been suggested to have a greater impact on biofilm resistance features than the limitation of harmful substances diffusion by the matrix structure [5]. In the matrix of *C. glabrata* biofilms have been found mainly carbohydrates and proteins, but lipids and nucleic acids were also described, though in lower quantities [6,7]. Matrix carbohydrates and lipids have been shown to be related with antifungal resistance of *C. glabrata* biofilms [6,8,9]. The function of matrix proteins is unclear but in *C. albicans* they were proposed to contribute to the delivery and organization of matrix carbohydrates, act as a digestive system, that provides a nutrient source for biofilm cells, and may be also related with the control of biofilm dispersion [10,11]. Importantly, although several studies have focused on the genetic regulation of *C. albicans* biofilm matrix [10–14] few have studied matrix regulation in *C. glabrata* [6,15]. An interesting biofilm matrix regulator found in *C. albicans* is the transcriptional factor Zap1, which was shown to be a negative regulator of glucan delivery to the biofilm matrix [12]. However, in *C. glabrata* the role of Zap1 in biofilm matrix production is unknown.

Targeted gene deletion is the most used gene manipulation technique to study gene function [16,17] and has allowed the identification of biofilm matrix regulators in *Candida* species [6,12]. The technique is based on the replacement of the target gene by a DNA fragment containing a selection marker (cassette), through the homologous recombination between the cassette and genomic sequences flanking the target gene [18]. The most used cassettes integrate nutritional markers, which allow a relatively straightforward construction of many knockout mutants in a reduced time [16,17]. However these cassettes can only be used in auxotrophic strains and the knockout mutant cannot be directly compared with its parental strain due to the presence of the biosynthetic marker [18,19]. In order to overcome these limitations, cassettes integrating drug-resistance markers have been

developed, including the *SAT1* flipper cassette that confers resistance to nourseothricin [18]. Cassettes with drug-resistance markers can be used in any strain and, as the marker can be excised after the gene deletion, the obtained mutants differ from their parental strain only by the absence of a functional target gene [18,20]. However gene deletion using drug-resistance markers has been limited because it is time consuming and requires complex genomic manipulation and specific optimization for the target gene [17]. Although the *SAT1*-flipping strategy was developed to be applied in *C. albicans* [20,21], which is a diploid organism, it can be adapted to be used in the haploid fungus *C. glabrata*.

This study aimed the characterization of biofilm matrix regulation by *C. glabrata* transcriptional factor Zap1, at acidic conditions that mimic the vaginal acidity, through the analysis of biofilms of a knockout mutant strain lacking the *ZAP1* gene, constructed using the *SAT1*-flipping strategy.

3.2 Materials and Methods

3.2.1 Construction of mutant strains

The reference strain *C. glabrata* ATCC 2001 was genetically manipulated in order to obtain two strains: a knockout mutant lacking the *ZAP1* gene (*zap1Δ*) and its complemented strain, containing the reintegrated gene (*zap1Δ::ZAP1*). To construct the mutant strains the *SAT1*-flipping strategy was applied as previously described for *C. albicans* [20,21], with some adaptations to be used in *C. glabrata*.

3.2.1.1 Assembly of the deletion cassette

For the construction of the deletion cassette *C. glabrata* ATCC 2001 was grown on Yeast Peptone Dextrose (YPD; Sigma-Aldrich, St Louis, MO, USA) agar plates for 48 h at 30°C and some colonies were used to inoculate YPD medium that was incubated overnight, under agitation (120 rev/min) at 30°C. Next, the genomic DNA was extracted from grown cells using a yeast DNA extraction kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. The DNA was used for the PCR amplification of sequences of approximately 500 base pairs (bp) length located upstream and downstream of *C. glabrata ZAP1* gene, with primers specifically designed for these sequences (Table AI.1- Annex I). In the primers of the upstream sequence were incorporated restriction sites for *Apa* I and *Xho* I enzymes, and in those of the downstream sequence for *Sac* II and

Sac I enzymes (Figure 3.1). After the amplification, the sequences were digested with the respective restriction enzymes (Thermo Scientific, Bremen, Germany) during 8 h, at the optimal temperature for each enzyme. A plasmid containing the *SAT1* flipper cassette (pSFS2) was digested with the same restriction enzymes leading to the excision of the cassette from the plasmid (with *Xho* I and *Sac* II) and the oblation of the remaining vector backbone (with *Apa* I and *Sac* I) (Figure 3.1). After the digestion of the flanking sequences and the plasmid, the DNA fragments obtained were separated by agarose gel electrophoresis, excised from the gel and purified using a gel DNA recovery kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. Then, a quadruple ligation was performed with the digested vector backbone, the cassette and the upstream and downstream fragments, by incubating them with T4-DNA ligase and ligation buffer (Thermo Scientific, Bremen, Germany) overnight at 16°C. Due to the compatible extremities obtained after the digestions this process generate a plasmid construct formed by the *SAT1* flipper cassette with the upstream and downstream *ZAP1* sequences cloned in its left and right borders, respectively (Figure 3.1).

The plasmid was used to transform competent *Escherichia coli* cells by incubating it with them for 30 min in an ice-cold buffer containing calcium chloride, followed by 45 s at a 42°C and 2 min on ice. After transformation, the cells were diluted into Super Optimal broth with Catabolite repression (SOC; Sigma-Aldrich, St Louis, MO, USA), and incubated at 37°C under shaking for 1 h. Transformed cells were plated onto Lysogeny Broth (LB; Sigma-Aldrich, St Louis, MO, USA), agar plates containing ampicillin to select positive transformants that incorporated the plasmid. The plasmid was extracted from *E. coli* cells using a mini-prep kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. Next, the constructed plasmid was checked through the amplification of the deletion cassette and some of its parts, followed by agarose gel electrophoresis to confirm the fragments' size (the primers designed for the confirmations are presented in Table AI.1 – Annex I). Additionally, the construct was also checked by restriction enzyme digestion (using the same restriction enzymes as previously used in the deletion cassette assembly), followed by fragments' size confirmation using agarose gel electrophoresis. After the confirmation that a plasmid containing the correct construct was obtained, the complete deletion cassette was excised from the plasmid, by digesting the plasmid with *Apa* I and *Sac* I enzymes. The obtained fragments were separated by agarose gel electrophoresis and the fragment corresponding to the deletion cassette was extracted from the gel, purified and dissolved in distilled water. The excision of the cassette from the constructed plasmid was repeated until a quantity of 2 µg of deletion cassette was achieved.

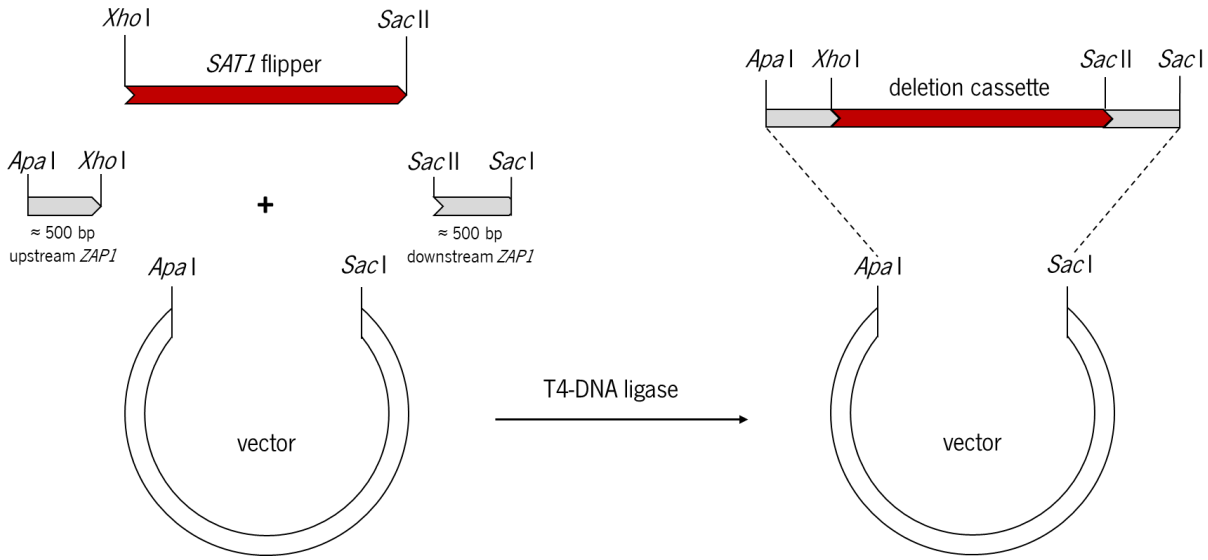


Figure 3.1 Assembly of the deletion cassette. Schematic representation of the quadruple ligation performed for cloning the upstream and downstream sequences of *ZAP1* into a plasmid containing the *SAT1* flipper cassette, generating a plasmid construct containing the deletion cassette.

3.2.1.2 Transformation of *C. glabrata* cells

Candida glabrata ATCC 2001 cells grown in YPD agar (as described in section 3.2.1.1) were incubated in YPD medium overnight at 30°C under agitation (120 rev/min), re-inoculated in fresh YPD medium and were again incubated overnight at 30°C under agitation. The cells were collected by centrifugation and resuspended in distilled water. The suspension was mixed with Tris-EDTA (TE) buffer and lithium acetate (Sigma-Aldrich, St Louis, MO, USA), and incubated for 1 h at 30°C under agitation (120 rev/min). Next, Dithiothreitol (DTT; Thermo Scientific, Bremen, Germany) was added to the suspension, which was incubated for further 30 min at 30°C under agitation. The cells were centrifuged and washed sequentially in ice-cold water and ice-cold sorbitol, and finally resuspended in sorbitol. The cell suspension was then mixed with the purified DNA fragment containing the deletion cassette (described in section 3.2.1.1), in a pre-cooled electroporation cuvette and incubated 5 min on ice. The cuvette was placed into an electroporator and the cells were electroporated at 1.8 kV. This process allowed the cassette to enter the cells and integrate *C. glabrata* genome by replacing *ZAP1* gene through the homologous recombination of the flanking sequences of the cassette and the flanking sequences of *ZAP1* gene (Figure 3.2). After electroporation, cells were washed in sorbitol, resuspended in YPD medium and incubated for 4 h at 30°C under agitation (120 rev/min). Next, the suspensions

were spread on YPD agar plates containing nourseothricin (200 mg/l), and incubated 48 h at 30°C, to select the nourseothricin-resistant transformants that have integrated the cassette. The selected transformants were suspended in Yeast Peptone Maltose (YPM) medium and incubated at 30°C under shaking, in order to perform a maltose-induced excision of the cassette. Next, the cells were spread on YPD agar plates containing a low concentration of nourseothricin (20 mg/l) (Thermo Scientific, Bremen, Germany) and incubated 48 h at 30°C. Cells that have lost the *SAT1* flipper cassette became nourseothricin-sensitive and appeared as small colonies, whereas cells that have retained the cassette formed large colonies. To confirm that small colonies corresponded to the knockout mutant desired (*zap1Δ*), the absence of the *ZAP1* gene and of the cassette were confirmed by PCR amplification and the genome was also sequenced.

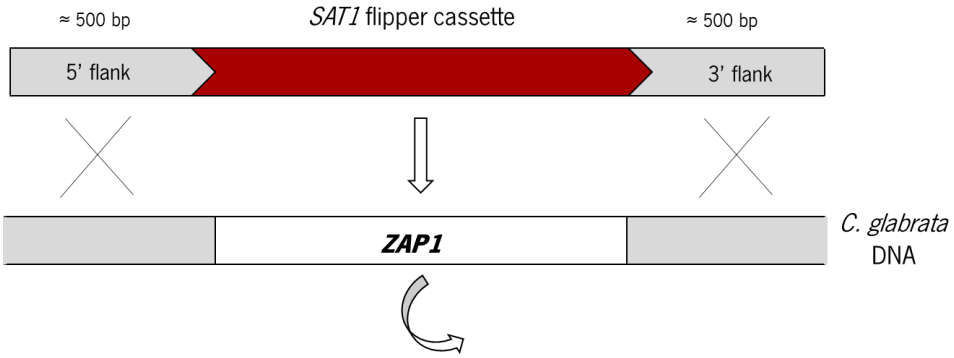


Figure 3.2 Insertion of *SAT1* flipper cassette into *Candida glabrata* genome. Schematic representation of the homologous recombination between the flanking sequences of the deletion cassette and the sequences flanking the *ZAP1* gene in *C. glabrata* genome, leading to the replacement of *ZAP1* gene by the *SAT1* flipper cassette.

3.2.1.3 Construction of the complemented strain

In order to complement the knockout mutant strain by re-integrating the deleted *ZAP1* gene, all the procedures described in sections 3.2.1.1 and 3.2.1.2 were performed but using the knockout mutant (*zap1Δ*) for the transformation instead of *C. glabrata* ATCC 2001. Additionally, the deletion cassette assembly was adapted in order to include not only the flanking sequences of the *ZAP1* gene but also the gene itself. The obtained complemented strain (*zap1Δ::ZAP1*) was confirmed by PCR amplification and sequencing (the primers designed to the assembly of the complemented deletion cassette and the confirmations of the construct are presented in Table AI.1 – Annex I).

3.2.2 Mutant strains' analysis

In this study, the growth and virulence related features of the two constructed strains (*zap1Δ* and *zap1Δ::ZAP1*) and the reference strain *C. glabrata* ATCC 2001 were analysed.

3.2.2.1 Initial culture conditions

The strains were initially grown in Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany), for 48 h, followed by 18 h of growth in Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany), as described in Chapter 2 - section 2.2.1. For the subsequent planktonic growth or biofilm development, the cells were suspended in Roswell Park Memorial Institute (RPMI; Sigma-Aldrich, St Louis, MO, USA) medium settled to pH 4 (with lactic acid), being the cellular density adjusted to $1x10^5$ cells/ml using a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany). The experiments described in next sections were performed in triplicate (same pre-inoculum) and in three independent assays (pre-inoculums independently prepared).

3.2.2.2 Planktonic growth analysis

For the planktonic growth, 25 ml of the cellular suspensions prepared in RPMI at pH 4, were incubated for 24 h at 37°C under agitation (120 rev/min). The optical density of the suspensions (at 690 nm) was measured over time using a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, VT, USA). Additionally, the number of cultivable cells after 24 h of growth was determined by colony forming units (CFU) counting methodology. Briefly, a serial of 10-fold dilutions from the planktonic suspensions were prepared in Phosphate Buffered Saline (PBS), that were plated on SDA and incubated for 24 h at 37°C. After incubation, the number of CFUs was counted and the results were presented as Log CFUs/ml.

3.2.2.3 Biofilm formation and analysis

For the development of biofilms, the cellular suspensions prepared in RPMI at pH 4 were placed into wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) (200 µl per well) and incubated at 37°C under agitation (120 rev/min) for 24 h [7]. Biofilms were washed with 200 µl of PBS to remove non-adherent cells and analysed.

The biofilms' total biomass was analysed by Crystal Violet (CV) staining methodology [7] and the number of cultivable cells into biofilms by CFU counting methodology [22], as described in Chapter 2 - sections 2.2.4. and 2.2.5, respectively. The results were presented per unit area of biofilm. Confocal laser scanning microscope (CLSM; Olympus BX61, Model FluoView 1000, Portugal) was used to analyse the biofilms' structure, as described in Chapter 2 - section 2.2.6.

3.2.2.4 Planktonic supernatant and biofilm matrix analysis

For these analyses planktonic cells and biofilms were grown as indicated above (sections 3.2.2.2 and 3.2.2.3), but the biofilms were developed in 24-wells plates (1 ml of suspensions per well). The biofilms were washed, scrapped from the wells with PBS and separated from their matrices by sonication followed by centrifugation, as described in Chapter 2 - section 2.2.8. Additionally, the planktonic cells were removed from their supernatants by centrifugation at 5000 g for 5 min at 4°C. The suspensions containing the matrices and planktonic supernatants were filtered through a 0.2 μm nitrocellulose filter. Then, their contents of protein, total carbohydrate and (1,3)-β-D-glucan were measured, using the BCA Kit [7], phenol-sulfuric method [23] and Glucatell (1,3)-β-D-Glucan Detection Reagent kit [24], respectively, as described in Chapter 2 - sections 2.2.8.1, 2.2.8.2 and 2.2.8.3. The results of the components of planktonic supernatants were presented per ml of supernatant, and those of the matrices per g of the biofilm cells' dry weight, which was achieved by drying, at 37°C, the biofilm cells separated from the matrices, until a constant weight was obtained [9].

Additionally, biofilm matrix proteins were identified by nano Liquid chromatography-tandem mass spectrometry (LC-MS/MS), in an equipment composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) [25], as fully detailed in Chapter 2 - section 2.2.8.1. The identification of matrix proteins and the assessment of their potential biological role were performed using UniProt and *Candida* Genome Database [26,27].

3.2.2.5 Susceptibility assays

To analyse the susceptibility of biofilms to caspofungin (Merck Sharp & Dome, Paço de Arcos, Portugal), biofilms were formed as described above (section 3.2.5). After 24 h of biofilm formation, the medium was aspirated and solutions with 0, 1, 4 and 10 mg/l of caspofungin, in RPMI at pH 4, were

added to the pre-formed biofilms (200 µl per well), that were incubated for further 24 h at 37°C under agitation. Next, the biofilms were washed with PBS and their biomasses were analysed by CV staining methodology, as described in Chapter 2- section 2.2.4. The results were presented as the percentage of biomass reduction in relation to the biomass of biofilms formed in the absence of caspofungin. Additionally, in order to choose the concentrations of caspofungin tested in biofilms, the minimum inhibitory concentration (MIC) of caspofungin was previously assessed, for all strains, using the CLSI M27 reference protocol [28].

3.2.2.6 Measurement of *ZAP1* transcript by quantitative real-time PCR (qRT-PCR)

The expression levels of *ZAP1* gene in *C. glabrata* ATCC 2001 planktonic and biofilm cells were assessed using qRT-PCR. The experimental setups used to cultivate the cells in planktonic and biofilm life styles were the same as described above (using 24-well polystyrene microtiter plates). Developed biofilms were scraped from the plates with PBS and the suspensions were sonicated for 30 s at 30 W, to separate the cells from the biofilm matrix [7]. Then, all cell suspensions (planktonic and biofilm cells) were centrifuged at 3000 g for 10 min at 4°C, the supernatants were rejected and pellets were used for RNA extraction, that was performed using RiboPure – Yeast Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Next, 0.5 µg of total RNA collected from each sample was used to obtain the complementary DNA (cDNA), using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. cDNA synthesis was performed at 70°C for 5 min followed by 42°C for 1 h and lastly 5 min at 95°C to stop the reaction.

Approximately 125 ng of the synthesized cDNA were used for the qRT-PCR. qRT-PCR (CF X96 Real-Time PCR System; Bio-Rad, Hercules, CA, USA) was used to determine the relative levels of mRNA transcripts, with the transcript level of *ACT1* mRNA applied as an internal control. Primers for *ZAP1* and *ACT1* were designed using Primer3 web software and their sequences are provided in Table AI.1 – Annex I. Full-length gene sequences were obtained from *Candida* Genome Database (www.candidagenome.org) [29]. The sequence of each primer was compared to the *Candida* genome database using BLAST [30], to confirm their specificity. The specificity of each primer pair, for its corresponding target gene, was also confirmed, by applying a PCR to genomic DNA extracted from *C. glabrata* planktonic cells. The qRT-PCR was performed using reaction mixtures consisting of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), cDNA samples and the primer pair respective to the target gene (50 µM). Negative controls (dH_2O) and non-reverse transcriptase controls (NRT) were also included in each run. qRT-PCR was performed at 98°C for 2 min in the initial denaturation step,

followed by denaturation at 98°C for 5 s and primer annealing at 57°C for 5 s, during 40 cycles. In each cycle it was generated a melting curve running a dissociation stage at 60°C, to verify the amplification product specificity. Control samples were included on each plate to ensure that multiple plates could be compared. The cycle threshold (Ct) value of each sample was determined and the relative gene expression levels calculated using the ΔCt method [31], being normalized with the *ACT1* internal control gene ($Ct_{average}$ = 28.08 ± 0.39).

3.2.2.7 Statistical analysis

All the quantitative results obtained in this study were statistically analysed using GraphPad Prism 6 software. For that, the results obtained for the knockout mutant strain were compared with those of the complemented or wild type strains, using the t test analysis. All tests were performed with a confidence level of 95%.

3.3 Results

3.3.1 Mutant strains' construction

The *SAT1*-flipping strategy was successfully used to construct a *C. glabrata* knockout mutant lacking *ZAP1* gene (*zap1Δ*) and its complemented strain (*zap1Δ*::*ZAP1*). Figure 3.3 shows the fragments' size obtained after the amplification of *ZAP1* gene plus its flanking sequences, in the wild-type (*C. glabrata* ATCC 2001) and mutant strains. As desired, the *zap1Δ* strain does not contain the *ZAP1* gene, and thus only a fragment corresponding to its flanking sequences was amplified (≈1000bp). Additionally, the complemented strain presented an amplification similar to that of the wild-type strain, corresponding to the target gene size (2142 bp) plus its flanking sequences (≈1000 bp). The correct deletion and reintegration of *ZAP1* gene was also confirmed by sequencing.

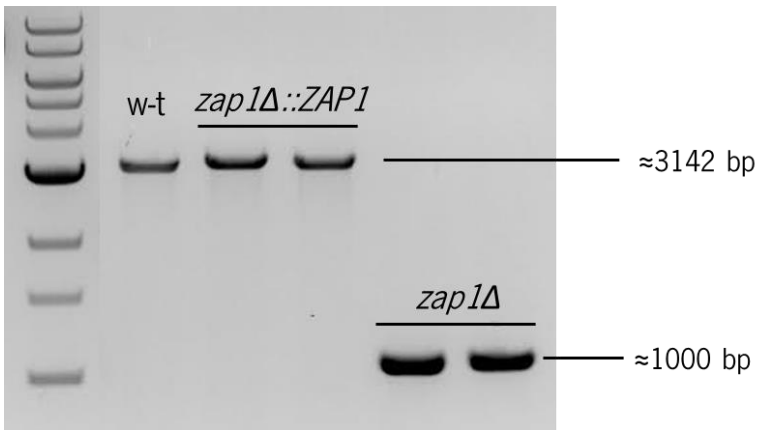


Figure 3.3 Fragments' size obtained after the amplification of *Candida glabrata ZAP1* gene. The amplification of *ZAP1* gene (2142 bp) plus its flanking sequences (≈1000 bp) was performed by PCR in *C. glabrata* ATCC 2001 (w-t), *zap1Δ* knockout mutant and its complemented strain *zap1Δ::ZAP1*.

3.3.2 Influence of *ZAP1* deletion on *C. glabrata* planktonic growth and biofilm formation

In order to analyse if the deletion of *ZAP1* influences *C. glabrata* ability to grow planktonically, free-floating cells of the knockout mutant (*zap1Δ*) and of its complemented (*zap1Δ*::*ZAP1*) and wild-type strains, were cultivated in RPMI buffered to pH 4 for 24 h. The results (Figure 3.4) showed that the three strains have similar growth curves (Figure 3.4-A) and the number of cultivable cells after 24 h of growth had no statistical differences between them (P-value >0.05) (Figure 3.4-B).

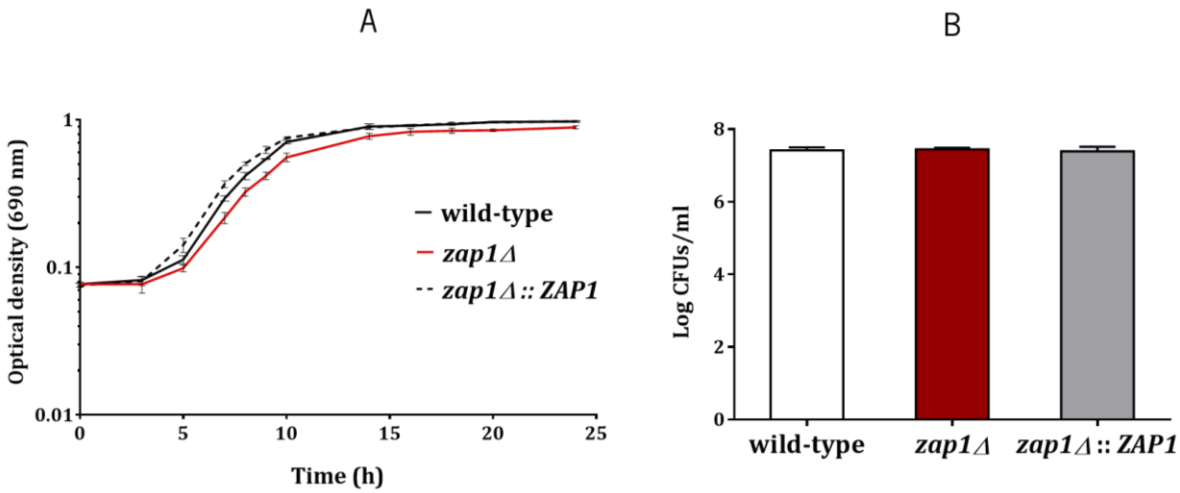


Figure 3.4 Influence of *ZAP1* deletion on *Candida glabrata* planktonic growth. **(A)** Growth curves (optical density) and **(B)** cultivable cells determination (Log CFUs/ml) of *C. glabrata* ATCC 2001 (wild-type), *zap1Δ*, and *zap1Δ::ZAP1* grown planktonically in RPMI at pH4. Error bars represent standard deviation.

In order to evaluate the influence of *ZAP1* deletion on *C. glabrata* biofilm formation, biofilms of *C. glabrata* ATCC 2001, *zap1Δ* and *zap1Δ*::*ZAP1* were developed for 24 h in RPMI at pH 4, and analysed in terms of total biomass (Figure 3.5-A) and cultivable cells (Figure 3.5-B). The biofilm of the knockout mutant showed a slight, but statistically significant (P-value ≤0.05), increase of total biomass compared to that of complemented or wild-type strains (Figure 3.5-A). Biofilm biomasses of the complemented and wild-type strains did not show difference between them. Additionally, the three tested strains presented similar number of cultivable cells with no statistical difference between them (Figure 3.5-B).

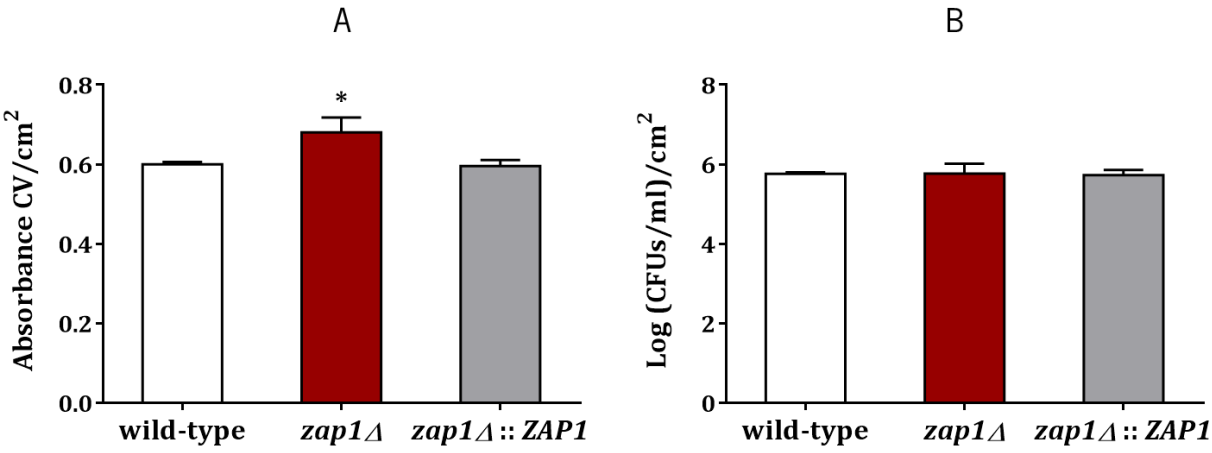


Figure 3.5 Effect of *ZAP1* deletion on *Candida glabrata* biofilm formation. (A) Total biomass quantification (Absorbance CV/cm²) and **(B)** cultivable cells determination (Log (CFUs/ml)/cm²) of *C. glabrata* ATCC 2001 (wild-type), *zap1Δ* (knockout mutant lacking *ZAP1* gene) and *zap1Δ*::*ZAP1*, (complemented strain) biofilms developed for 24 h in RPMI at pH 4. Error bars represent standard deviation. Asterisk represents statistical differences between the knockout mutant and other strains (* P-value ≤0.05).

The biofilms of the three strains were further visualized by CLSM in order to analyse the influence of *ZAP1* deletion on biofilm structure and thickness. The obtained images (Figure 3.6), show similar structures between the wild-type (*C. glabrata* ATCC 2001) and complemented (*zap1Δ::ZAP1*) strains. In contrast, the biofilm of z*ap1Δ* strain presented a different structure, that seems more cohesive and with a glistening appearance (Figure 3.6). Additionally, the knockout mutant showed a slightly higher biofilm thickness (21.5 μm ± 3.0) compared to that of complemented (17.3 μm ± 1.2) or wild-type strains (18.2 μm ± 1.8), although not statistically significant (P-value >0.05).

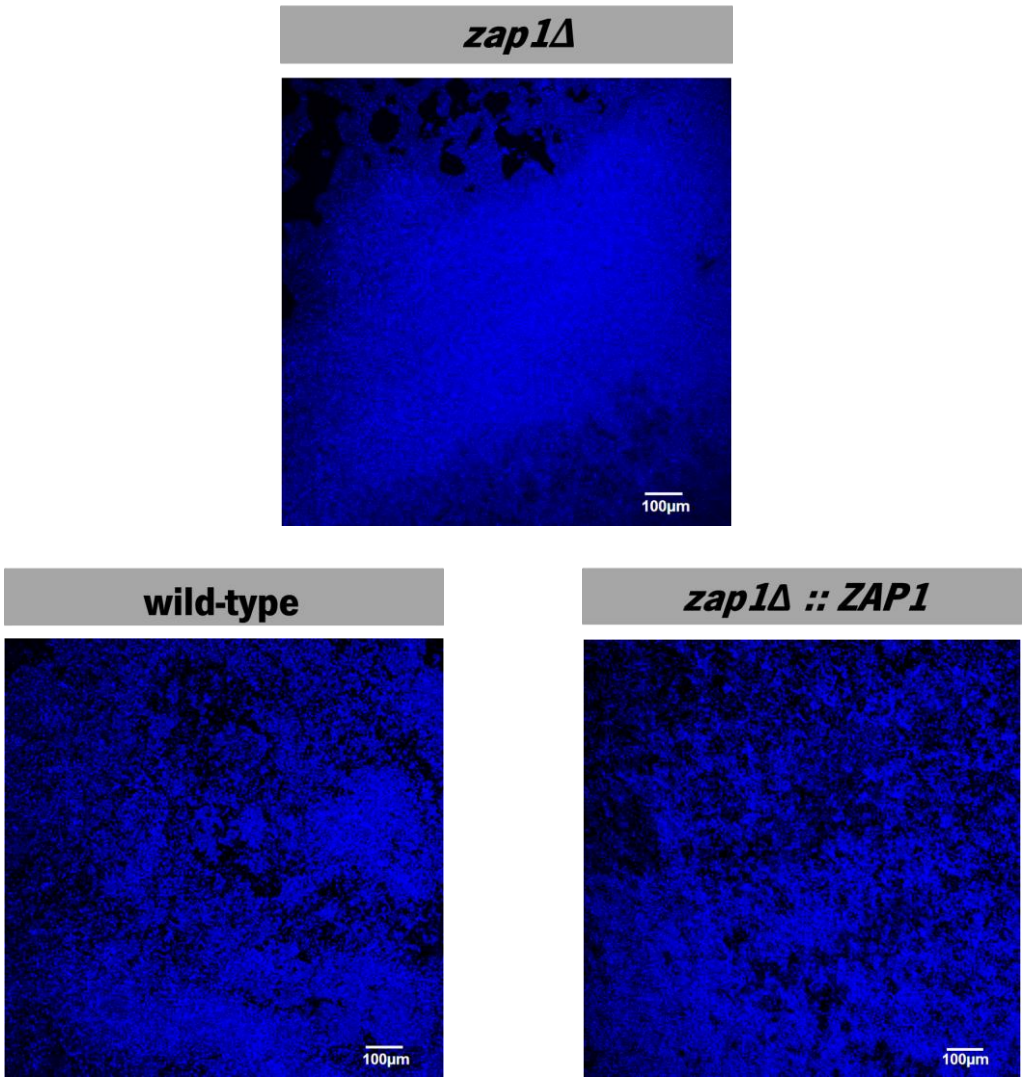


Figure 3.6 Effect of *ZAP1* deletion on *Candida glabrata* biofilm structure. Confocal laser microscopy images of *C. glabrata* ATCC 2001 (wild-type), *zap1Δ* (knockout mutant lacking *ZAP1* gene) and *zap1Δ::ZAP1*, (complemented strain) biofilms developed for 24 h in RPMI at pH 4. Original magnification was 10x and the scale bars correspond to 100 µm.

To deep the study of the role of Zap1 in biofilm formation the levels of expression of *ZAP1* gene in *C. glabrata* ATCC 2001 planktonic and biofilm cells grown 24 h in RPMI at pH 4 were also analysed, using qRT-PCR. The results (Figure 3.7) showed statistically lower (P-value ≤0.01) expression of *ZAP1* in biofilm than planktonic cells.

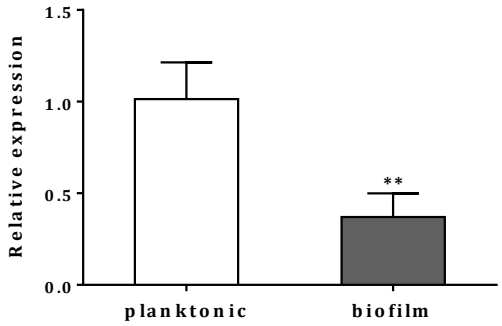


Figure 3.7 Expression of *ZAP1* gene in *Candida glabrata* planktonic and biofilm cells. Transcript levels of *ZAP1* gene, estimated by qRT-PCR, of *C. glabrata* ATCC 2001 planktonic and biofilms cells, cultivated 24 h in RPMI at pH 4. The values of the transcript levels were normalized using as internal control the levels of *ACT1* mRNA. Error bars represent standard deviation. Asterisks represent statistical difference between growth lifestyles (** P-value ≤0.01).

3.3.3 Role of *C. glabrata* Zap1 in biofilm matrix production

The matrices of the wild-type, *zap1Δ* and *zap1Δ::ZAP1* biofilms were analysed in order to evaluate the influence of *ZAP1* deletion on the production of the major matrix components, protein and carbohydrate (Table 3.1). The quantity of protein secreted to the matrix was statistically higher (P-value ≤0.01) in the biofilm of the knockout mutant strain compared to the other two strains, presenting almost the double of the quantity found in the complemented strain. The total carbohydrate content was also statistically higher (P-value ≤0.05) in the matrix of the *zap1Δ* biofilm, presenting more than the triple quantity detected in *zap1Δ::ZAP1* biofilm. Consistently, the (1,3)-β-D glucan was also increasingly (P-value ≤0.05) secreted to the matrix of the *zap1Δ* biofilm, although in lower extent in relation to the total carbohydrate increase.

Additionally, in cells grown planktonically (24 h in RPMI at pH 4) the secretion of the same components was also analysed. The results (Table 3.1) showed a similar trend to that observed in the biofilm matrix, but presenting only a slight increase that was not statically significant (P-value >0.05), of all components in the supernatant of *zap1Δ* planktonic cells.

Table 3.1 Influence of *ZAP1* deletion on *Candida glabrata* secreted components. Quantification of protein carbohydrate and (1,3)-β-D-glucan secreted to the planktonic supernatant or to the biofilm matrix by *C. glabrata* ATCC 2001 (wild-type), *zap1Δ* and *zap1Δ*::*ZAP1* strains, after 24 h of growth in planktonic or biofilm lifestyles in RPMI at pH 4. The results of biofilm matrix were normalized with the respective biofilm dry weight and all the results are presented as means ± standard deviations. Asterisks represent statistical differences between the knockout mutant and other strains (* P-value ≤0.05; ** P-value ≤0.01).

Sample	Component	Strain		
		wild-type	***zap1Δ***	***zap1Δ*:: *ZAP1***
Biofilm matrix	**Protein** (mg/g biofilm)	41.33 ± 4.56	87.16 ± 2.22**	45.44 ± 3.88
	Carbohydrate (mg/g biofilm)	94.08 ± 14.36	368.87 ± 59.03*	106.60 ± 19.62
	(1,3)-β-D-glucan (ng/g biofilm)	45.91 ± 1.16	62.24 ± 5.67*	46.33 ± 0.16
Planktonic supernatant	**Protein** (μg/ml)	108.85 ± 7.56	120.19 ± 3.40	112.41 ± 5.52
	Carbohydrate (mg/ml)	2.47 ± 0.14	3.16 ± 0.07	2.51 ± 0.24
	(1,3)-β-D-glucan (pg/ml)	104.27 ± 11.67	142.58 ± 13.94	103.71 ± 7.94

In order to evaluate the potential relation of the biofilm matrix results with the biofilms' resistance to caspofungin, biofilms grown for 24 h in RPMI at pH 4 were treated with caspofungin at 1, 4 and 10 mg/l and further developed for additional 24 h. The results (Table 3.2) revealed similar biofilm biomass reduction in the knockout mutant, complemented and wild-type strains, at all the concentrations of caspofungin tested. Consistently, the three strains presented similar MIC_{90} to caspofungin of approximately 0.5 mg/l.

Table 3.2 Influence of *ZAP1* deletion on *Candida glabrata* biofilm susceptibility to caspofungin. Percentage of biomass reduction of *C. glabrata* ATCC 2001 (wild-type), *zap1Δ* and *zap1Δ*::*ZAP1* biofilms grown for 24 h in RPMI at pH 4 and for additional 24 h in presence of caspofungin at 1, 4 and 10 mg/l. The percentage is relative to biofilms grown in absence of caspofungin. The results are presented as means ± standard deviations.

[caspofungin] mg/l	**% biofilm biomass reduction**		
	wild-type	***zap1Δ***	***zap1Δ*::*ZAP1***
1	0.00	0.00	0.00
4	12.96 ± 6.60	13.57 ± 5.22	15.02 ± 6.83
10	34.64 ± 5.04	38.61 ± 7.54	36.42 ± 5.93

3.3.4 Regulation of biofilm matrix proteins by *C. glabrata* Zap1

Firstly, the proteomic profile of *C. glabrata* ATCC 2001 biofilm matrix was analysed by mass spectrometry, revealing the presence of 397 different proteins. A subset of the most relevant proteins is presented in Table 3.3 and the entire list is presented in Table AI.2 – Annex I. In this group of proteins, 71 correspond to proteins specifically present in the matrix at acidic conditions (previously found in Chapter 2 - section 2.3.4, Figure 2.6), which are highlighted in bold in Table 3.3 and Table AI.2 – Annex I. The 397 proteins were clustered according to the biological processes in which they are involved, according to UniProt and *Candida* Genome Database (or predicted to be involved by orthology), and the results revealed an enrichment of "metabolism", "cell rescue, defence and virulence" and "protein synthesis" classes, and around 25% of proteins with unknown function (Figure 3.8). Among the most enriched classes, the subclasses "carbohydrate metabolism", "amino acid metabolism" and "stress response" were the most representative, accounting around 16% of total proteins each.

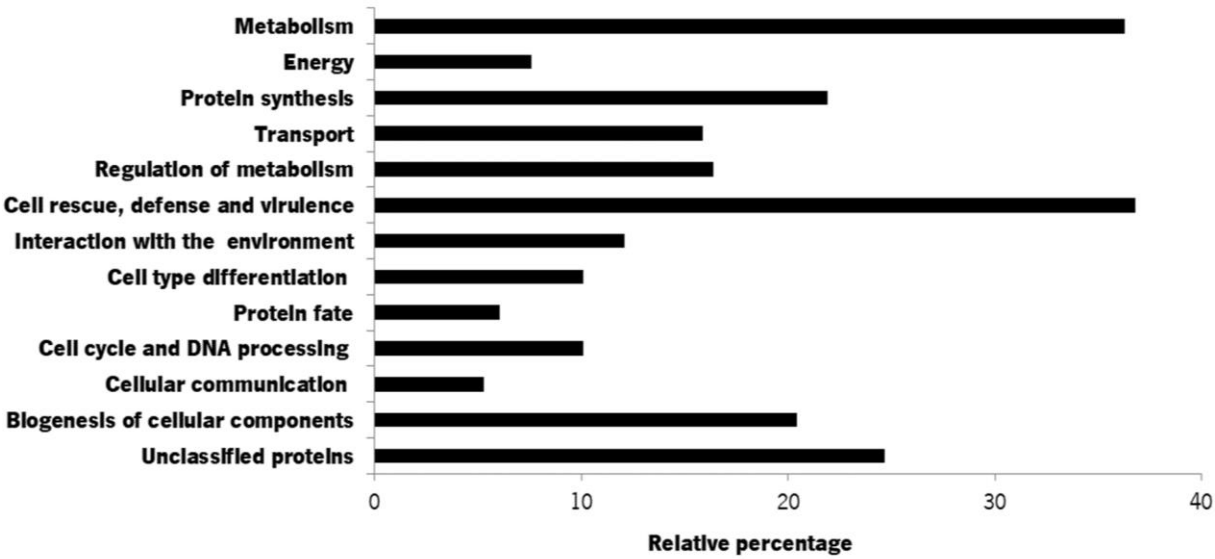


Figure 3.8 Functional distribution of *Candida glabrata* biofilm matrix proteins at acidic conditions. The proteins found in the matrix of *C. glabrata* ATCC 2001 biofilms developed for 24 h at pH 4 were clustered according to their biological function annotated in *Candida* Genome Database and UniProt. The percentages shown correspond to the number of proteins included in each functional class compared to the total number of proteins in the biofilm matrix.

Table 3.3 *Candida glabrata* biofilm matrix proteins at acidic conditions. A subset of proteins found in the matrix of *C. glabrata* ATCC 2001biofilms developed for 24 h at pH 4 are herein shown and the full list is available in Table AI.2 – Annex I. Proteins previously reported to be secreted by *C. glabrata* (or by *C. albicans* orthologs) are underlined and those found to be specifically secreted to the matrix at acidic conditions are highlighted in bold. The biological function indicated is based on the information available at *Candida* Genome Database and UniProt.

Protein	Biological function
Ygk3p	Predicted kinase activity; role in response to heat, salt stress and proteolysis
Yps5p	Putative aspartic protease; predicted GPI-anchor
Yps2p	Putative aspartic protease; predicted GPI-anchor, induced in response to low pH
Yps11p	Putative aspartic protease; predicted GPI-anchor, induced in response to low pH
Yps1p	Putative aspartic protease; predicted GPI-anchor, regulation of pH homeostasis at low pH
Vig9p	GDP-mannose pyrophosphorylase
Tir1p	Putative GPI-linked cell wall mannoprotein
Ssr1p	Putative GPI-linked cell wall protein
Ssb1p	Heat shock protein
Ssa3p	Heat shock protein of the HSP70 family
Sec14p	Predicted phosphatidylinositol binding activity
Scw4p	Putative transglycosidase with a predicted role in the modification of 1,3-beta-glucan
Scl1p	Alpha subunit of the 20S proteasome
Rho1p	Beta-1,3-glucan synthase regulatory subunit
Pma1p	Putative plasma membrane proton pump with a predicted role in pH homeostasis
Pdr13p	Protein with a predicted role in pleiotropic drug resistance
Pdr1p	Zinc finger transcription factor, regulates drug efflux pumps
Mp65p	Mannoprotein
Kre9p	Protein involved in cell wall beta-1,6-glucan synthesis
Hxt4p	Predicted glucose transmembrane transporter activity
Hsp31p	Putative cysteine protease
Hsp12p	Heat shock protein
Hsc82p	Putative heat shock protein
Gre2(B)p	Putative methylglyoxal reductase (NADPH-dependent)
Gas5p	Putative 1,3-beta-glucanosyltransferase
Gas4p	Putative 1,3-beta-glucanosyltransferase
Gas2p	Putative 1,3-beta-glucanosyltransferase
Gas1p	Putative 1,3-beta-glucanosyltransferase
Fks2p	Putative 1,3-beta-glucan synthase component
Epa3p	Epithelial adhesion protein; GPI-anchored
Epa2p	Epithelial adhesion protein; predicted GPI-anchor
Epa6p	Sub-telomerically encoded adhesin with a role in cell adhesion
Aed1p	Adhesin-like protein; predicted GPI anchor
Awp6p	Cell wall adhesin-like protein
Asc1p	40S small subunit ribosomal protein
Cagl0m05731gp	Predicted alpha-1,3-mannosyltransferase activity
Cagl0m08756gp	Putative exo-1,3-beta-glucanase; predicted GPI-anchor
Cagl0l07502gp	Putative GPI-linked cell wall protein
Cagl0l06424gp	Predicted GPI-linked adhesin-like protein
Cagl0l03289gp	Putative secreted beta-glucosidase
Cagl0j09922gp	Predicted glucan endo-1,3-beta-D-glucosidase activity
Cagl0f04873gp	Predicted glucosidase activity and role in (1,6)-beta-D-glucan biosynthetic process
Cagl0f03003gp	Putative osmosensor activity and role in (1,3)-beta-D-glucan biosynthetic process
Cagl0f00759gp	Putative mannosyltransferase

In order to determine the biofilm matrix proteins regulated by Zap1, the proteins present in the matrix of *zap1Δ* and *zap1Δ::ZAP1* biofilms were also identified by mass spectrometry. The proteins found in the wild-type and *zap1Δ*::*ZAP1* biofilms' matrices presented high similarity and for the further analyses of *zap1Δ::ZAP1* strain, only proteins commonly found in this and wild type strains were considered.

The comparison of proteins found in *zap1Δ* and *zap1Δ::ZAP1* biofilm matrices revealed a significant number of proteins commonly present in both strains (266) but there were also 31 and 134 proteins specifically present or absent in *zap1Δ* biofilm matrix, respectively. The 31 proteins specifically present in the biofilm matrix of the mutant strain are listed in Table AI.3 – Annex I, and the 134 specifically absent correspond to the first 134 proteins of Table AI.2 – Annex I (subsets of these proteins are presented in Figure 3.9). The two sets of proteins were clustered according to the predicted biological processes annotated in UniProt and *Candida* Genome Database. Among the 134 proteins found to be absent in the knockout mutant matrix, the functional classes with the highest enrichment were "stress response" (18%), "regulation of biological process" (14%) and "transport" (13%) (Figure 3.9). Other functional classes with a considerable representation include "response to chemical" and "translation". This set presented around 12% of proteins with unknown function. Among the 31 proteins found to be specifically present in the knockout mutant matrix, a significant number have unknown function (45%) and the most enriched functional classes were "adhesion" (19%) and "organelle organization" (16%) (Figure 3.9).

A schematic representation of the suggested regulation of *C. glabrata* biofilm matrix proteins by Zap1 is shown in Figure 3.9.

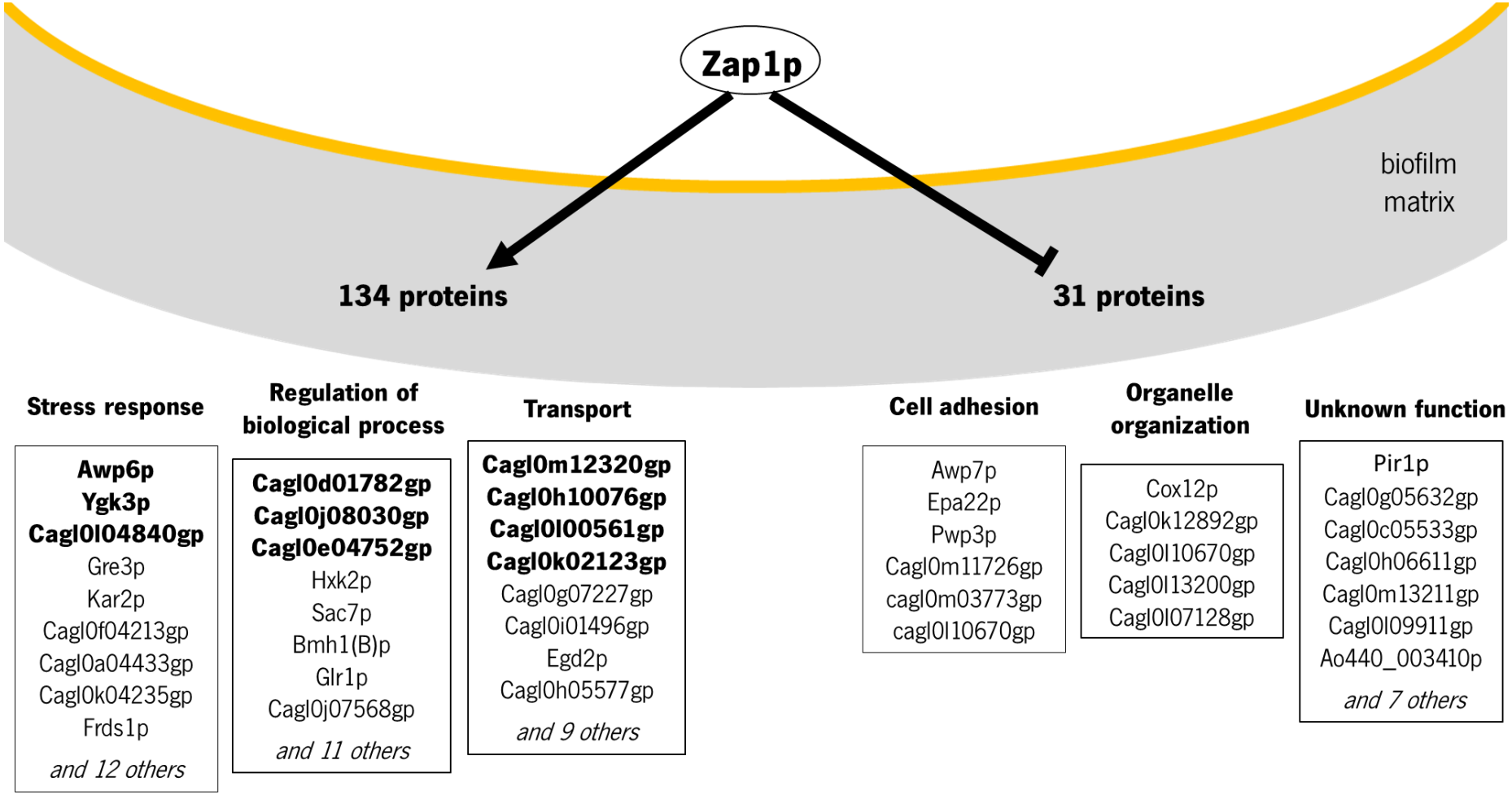


Figure 3.9 Regulation of biofilm matrix proteins by *Candida glabrata* Zap1. Schematic suggestion of matrix proteins regulation by Zap1 in *C. glabrata* biofilms developed for 24 h at pH 4. Zap1 is suggested to specifically induce the secretion of 134 proteins and to block the secretion of 31 proteins to the biofilm matrix. The three most enriched functional classes of each group of proteins are represented. The biological functions were obtained from UniProt and *Candida* Genome Database. Proteins specifically secreted to the matrix at acidic conditions are highlighted in bold.

3.4 Discussion

Candida glabrata is the second most common *Candida* species in the vaginal tract but one of the most challenging, due to its intrinsically high resistance to antifungals and high adaptability to stress conditions [32–34]. These features have a potential relation with its specific regulation of relevant virulence factors, as biofilms, which has been less studied comparatively to *C. albicans*. In this study it was aimed to undercover some relevant features of *C. glabrata* biofilms at acidic conditions, specifically their regulation by Zap1, a transcription factor with unknown function in *C. glabrata*, that was shown to regulate biofilm matrix production in *C. albicans* [12]. For this study a knock out mutant of *C. glabrata* ATCC 2001 lacking the *ZAP1* gene was constructed using the *SAT1* -flipping strategy, which allows high reliability in the comparison of the *zap1Δ* mutant with its complemented strain and the confirmed similarity between this and wild-type strains.

The deletion of *ZAP1* gene was found not to be affecting the growth rate or cultivability of *C. glabrata* planktonic cells (Figure 3.4). Additionally, biofilm cells cultivability was not also affected by the absence of this gene (Figure 3.5-B). In accordance, a large-scale study using a *C. glabrata* deletion collection did not find altered biofilm formation ability in the mutant lacking *ZAP1* gene [17] and in *C. albicans*, the deletion of this gene (alias *CSR1*) was reported not to alter the biofilm dry weight [12]. Interestingly, a slight increase of total biomass (Figure 3.5-A) and a glistening appearance (Figure 3.6) was observed in *zap1Δ* biofilm, suggesting a potential higher accumulation of matrix components. Consistently, higher quantity of the major matrix components, carbohydrate and protein, was found in the knockout mutant biofilm (Table 3.1). This result is suggestive of a negative regulation of *C. glabrata* biofilm matrix production by Zap1, in accordance to that reported for *C. albicans* [12]. Additionally, *C. glabrata* planktonic cells of *zap1Δ* mutant showed only a slight tendency (not statistically different) to secrete higher quantity of the compounds found in the biofilm matrix (Table 3.1), also as reported for *C. albicans* [12]. This specific and negative effect on matrix production may partially explain the lower expression of *ZAP1* gene that was found in the biofilm of the wild type strain compared to planktonic cells (Figure 3.7). In *C. albicans* a large-scale study found increased expression of *ZAP1* gene in biofilm than planktonic cells [35], however it was proposed that a decrease of *ZAP1* expression occurs during the process of biofilm maturation [12].

Among the matrix components analysed, the total carbohydrate presented the highest increase in *zap1Δ* biofilm (Table 3.1). Consistently, the carbohydrate component (1,3)-β-D-glucan was also found to be negatively regulated by Zap1 but its increase on the mutant biofilm was much lower in relation to that of total carbohydrate, suggesting that other carbohydrate components may be also affected by

ZAP1 deletion. The regulation of (1,3)-β-D-glucan matrix content by Zap1 is consistent with that reported for *C. albicans*, although the impact of *ZAP1* deletion on this matrix component seems to be higher in *C. albicans* [12]. Due to the higher matrix carbohydrate content detected in *zap1Δ* mutant, including of (1,3)-β-D-glucan, which have been shown to be related with antifungal resistance, it was hypothesized a potential relation with caspofungin resistance, an antifungal that exert its activity by inhibiting (1,3)-β-D-glucan synthases. However, the resistance to caspofungin was not affected in the knockout mutant biofilm (Table 3.2), and similar result was obtained for the MIC_{90} of this strain, suggesting that the regulation of matrix by Zap1 may not be directly related with biofilm resistance to antifungals at acidic conditions.

An interesting finding in *C. glabrata* matrix composition was a significant impact of *ZAP1* deletion on its protein content (Table 3.1). The biofilm matrix proteins and their regulation have been largely understudied in *C. glabrata*. In fact, in contrast with *C. albicans*, the *Candida* Genome Database does not have any proteins annotated in *C. glabrata* species as "biofilm matrix localization" or "extracellular localization", in manually curated or high-throughput annotation typologies [27]. This lack of knowledge instigated a more detailed analysis of *C. glabrata* biofilm matrix proteins using an advanced proteomic technique.

In the biofilm matrix of the wild-type strain were found 397 different proteins (Table AI.2 – Annex I) with enrichment of the functional classes "carbohydrate metabolism", "amino acid metabolism" and "stress response" (Figure 3.8). To the author's knowledge, this is the first study reporting the proteomic profile of *C. glabrata* biofilm matrix. Importantly, 73 matrix proteins have a predicted Glycosylphosphatidylinisotol- (GPI-) anchor (Table 3.3), which has been suggested to make proteins, especially those of the cell wall, more likely to be tethered to the extracellular portion of biofilm cells [11]. Nevertheless, a high quantity of proteins does not contain predicted secretion sequences, what suggests a non-secretion pathway or accumulation of proteins after cell death [10,36,37].

According to the information of *Candida* Genome Database [27], 95 of the 397 proteins are encoded by genes whose orthologs encode proteins previously found in *C. albicans* biofilm matrix or that are secreted by this species (underlined in Table 3.3 and Table AI.2 – Annex I). Additionally, a study of *C. albicans* biofilm matrix, similar to ours, found an abundance of proteins from carbohydrate and amino acid metabolism pathways [10]. As such, our study suggests some similarities between *C. glabrata* and *C. albicans* biofilm matrix proteome. Importantly, several cell wall proteins were found in *C. glabrata* matrix (69 proteins), with an abundance of hydrolases and transferases, including some enzymes predicted to be involved in (1,3)-β-glucan modification, as Gas1p, Gas2p, Gas4p, Gas5p,

Fks2, Scw4p, Rho1, Cagl0m08756gp and Cagl0j09922gp [26,27] (Table 3.3). In *C. albicans*, secreted cell wall glucanases and glucan transferases, have been shown to be involved in the delivery of (1,3)-β-glucan to the biofilm matrix [10,11], being suggested to release it from the cell wall, to organize it in the biofilm matrix [10] and potentially to degrade it as an energy source [11]. As such, similar roles may be suggested for *C. glabrata* biofilm matrix proteins. Additionally, some proteins involved in mannose modification were also found, including Vig9p, Tir1p, Mp65p, Cagl0f00759gp and Cagl0m05731gp [27] (Table 3.3). This result suggests that matrix proteins may also regulate the delivery and organization of mannans, which have been reported to be a major component of biofilm matrix carbohydrate [10]. Importantly, two proteins involved in glucan/mannans modification are predicted to have a role in *C. glabrata* biofilm formation (Mp65p and Cagl0j09922gp) [27]. Additionally, other proteins involved in *C. glabrata* biofilm formation and/or adhesion were also found in the matrix, including the epithelial adhesins Epa2, Epa3 and Epa6 [38,39] (Table 3.3).

An enrichment of stress response proteins was also found in *C. glabrata* biofilm matrix, what was an interesting result, as in *C. albicans* only some stress related proteins, mainly heat shock proteins, have been reported in the matrix [10]. This result suggests a species-specific regulation of *C. glabrata* matrix proteins and/or influence of the acidic conditions used in this study. In fact, among the 71 proteins found to be specifically secreted to the matrix at acidic conditions (highlighted in bold in Table AI.2 – Annex I), the stress response function was the most representative. Importantly, some of the acidic-specific proteins were previously reported to be involved in *C. glabrata* pH homeostasis at low pH, including the aspartic proteases Yps2 and Yps5 [40]. These results suggest a significant contribution of the environmental pH to the proteomic profile of *C. glabrata* biofilm matrix.

The analysis of *C. glabrata* matrix proteins potentially regulated by Zap1 revealed that 134 proteins have their matrix delivery induced by Zap1 and 31 proteins blocked by this transcription factor (Figure 3.9). This result indicates that besides being a negative regulator of matrix protein quantity, Zap1 also regulates the matrix proteomic profile, potentially increasing matrix proteins heterogenicity.

The majority of the 31 proteins that have a matrix delivery suggested to be blocked by Zap1 have unknown function (Table AI.3 – Annex I and Figure 3.9). Interestingly, 9 of those proteins (≈30%) were predicted cell wall proteins, most with a putative function in cell adhesion (Figure 3.9), and one protein with a predicted involvement in (1,6)-β-glucan modification (Cagl0I10670gp) [26]. Additionally, 2 other proteins with a predicted involvement in carbohydrate metabolism were also found (Cagl0h03993gp and Ao440_001871p) [26,27]. As discussed above, matrix proteins have a potential role in the delivery of matrix carbohydrates and cell wall proteins have been suggested to intermediate

the covalent linkages between the different exopolysaccharides, similar to what occurs in the cell wall [4,41]. As such, the results suggest that the negative regulation of matrix carbohydrate by Zap1 has a potential relation with its negative regulation of some matrix proteins. Although the regulation of matrix proteome by Zap1 in *C. albicans* is unknown, this transcription factor was shown to negatively regulate the expression of genes encoding proteins involved in cell wall structure and carbohydrate metabolism, including two extracellular glucoamylases (Gca1 and Gca2) suggested to promote matrix production by hydrolytic release of glucan fragments [12].

The Zap1 was also found to induce the delivery of 134 proteins to *C. glabrata* biofilm matrix (first 134 proteins of Table AI.2 – Annex I). The most enriched functional class among these proteins was "stress response", followed by "regulation of biological process" and "transport" (Figure 3.9). Interestingly, two predicted zinc-regulated proteins were found in this set (Ao440_000932p and Ao440_000495p) [26], what is in accordance with the Zap1 induction of genes involved in zinc homeostasis reported in *C. albicans* biofilms [12]. Importantly around 37% of the 71 proteins specifically secreted to the matrix at acidic conditions were found to have their delivery to the matrix induced by Zap1 (highlighted in bold in Table AI.2 – Annex I and Figure 3.9). This result suggests that the transcriptional factor Zap1 may have an intermediate role in the modulation of *C. glabrata* matrix proteome by acidic conditions.

This study reveals that the transcriptional factor Zap1 is a negative regulator of *C. glabrata* biofilm matrix production and modulates its proteomic profile. This report is the first to decipher the complex proteome of *C. glabrata* matrix but additional functional analyses will be important to discern the role of matrix proteins and clarify the relevance of their regulation by Zap1.

References

[1] **Amouri I, Sellami H, Borji N, et al.** Epidemiological survey of vulvovaginal candidosis in Sfax, Tunisia. *Mycoses* (2011); 54(5):e499-505.

[2] **Grigoriou O, Baka S, Makrakis E, et al.** Prevalence of clinical vaginal candidiasis in a university hospital and possible risk factors. *Eur J Obstet Gynecol Reprod Biol* (2006); 126(1):121–5.

[3] **Nobile CJ, Mitchell AP**. Microbial biofilms: e pluribus unum. *Curr Biol* (2007); 17(10):R349–53.

[4] **Pierce C, Vila T, Romo J, et al.** The *Candida albicans* biofilm matrix: composition, structure and function. *J Fungi* (2017); 3(1):14.

[5] **Al-Fattani MA, Douglas LJ**. Penetration of *Candida* biofilms by antifungal agents. *Antimicrob Agents Chemother* (2004); 48(9):3291–7.

[6] **Dominguez E, Zarnowski R, Sanchez H, et al.** Conservation and divergence in the *Candida* species biofilm matrix mannan-glucan complex structure, function, and genetic control. *MBio* (2018); 9(2).

[7] **Silva S, Henriques M, Martins A, et al.** Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* (2009); 47(7):681–9.

[8] **Rodrigues CF, Rodrigues ME, Henriques M**. Susceptibility of *Candida glabrata* biofilms to echinocandins: alterations in the matrix composition. *Biofouling* (2018); 34(5):569–78.

[9] **Rodrigues CF, Gonçalves B, Rodrigues ME, et al.** The effectiveness of voriconazole in therapy of *Candida glabrata*'s biofilms oral infections and its influence on the matrix composition and gene expression. *Mycopathologia* (2017).

[10] **Zarnowski R, Westler WM, Lacmbouh GA, et al.** Novel entries in a fungal biofilm matrix encyclopedia. *MBio* (2014); 5(4):e01333-14.

[11] **Taff HT, Nett JE, Zarnowski R, et al.** A *Candida* biofilm-induced pathway for matrix glucan delivery: implications for drug resistance. *PLoS Pathog* (2012); 8(8):e1002848.

[12] **Nobile CJ, Nett JE, Hernday AD, et al.** Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol* (2009); 7(6):e1000133.

[13] **Nett JE, Crawford K, Marchillo K, et al.** Role of Fks1p and matrix glucan in *Candida albicans* biofilm resistance to an echinocandin, pyrimidine, and polyene. *Antimicrob Agents Chemother* (2010); 54(8):3505–8.

[14] **Nett JE, Sanchez H, Cain MT, et al.** Genetic basis of *Candida* biofilm resistance due to drug-sequestering matrix glucan. *J Infect Dis* (2010); 202(1):171–5.

[15] **Rodrigues CF, Vilas Boas D, Haynes K, et al.** The *MNN2* gene knockout modulates the antifungal resistance of biofilms of *Candida glabrata*. *Biomolecules* (2018); 8(4):130.

[16] **Noble SM, Johnson AD**. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell* (2005); 4(2):298–309.

[17] **Schwarzmüller T, Ma B, Hiller E, et al.** Systematic phenotyping of a large-scale *Candida glabrata* deletion collection reveals novel antifungal tolerance genes. *PLoS Pathog* (2014); 10(6):e1004211.

[18] **Xu Q-R, Yan L, Lv Q-Z, et al.** Molecular genetic techniques for gene manipulation in *Candida albicans*. *Virulence* (2014); 5(4):507–20.

[19] **Lay J, Henry LK, Clifford J, et al.** Altered expression of selectable marker URA3 in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. *Infect Immun* (1998); 66(11):5301–6.

[20] **Sasse C, Morschhäuser J**. Gene deletion in *Candida albicans* wild-type strains using the *SAT1*-flipping strategy. *Methods Mol. Biol.*, vol. 845. (2012). pp. 3–17.

[21] **Reuß O, Vik A, Kolter R, et al.** The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans. Gene* (2004); 341:119–27.

[22] **Silva S, Henriques M, Oliveira R, et al.** *In vitro* biofilm activity of non-*Candida albicans Candida* species. *Curr Microbiol* (2010); 61(6):534–40.

[23] **DuBois M, Gilles KA, Hamilton JK, et al.** Colorimetric method for determination of sugars and related substances. *Anal Chem* (1956); 28(3):350–6.

[24] **Ferreira C, Gonçalves B, Vilas Boas D, et al.** *Candida tropicalis* biofilm and human epithelium invasion is highly influenced by environmental pH. *Pathog Dis* (2016); 74(8):ftw101.

[25] **Michalski A, Damoc E, Hauschild J-P, et al.** Mass spectrometry-based proteomics using Q exactive, a high-performance benchtop Quadrupole Orbitrap mass spectrometer. *Mol Cell Proteomics* (2011); 10(9):M111.011015.

[26] UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res* (2019); 47(D1):D506–15.

[27] **Skrzypek MS, Binkley J, Binkley G, et al.** The *Candida* Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data. *Nucleic Acids Res* (2017); 45(D1):D592–6.

[28] **CLSI**. *Reference method for broth dilution antifungal susceptibility testing of yeasts*. 4th ed. CLSI standard M27. Wayne, PA: Clinical and Laboratory Standards Institute; (2017).

[29] **Skrzypek MS, Arnaud MB, Costanzo MC, et al.** New tools at the *Candida* Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res* (2010); 38(Database issue):D428-432.

[30] **Altschul S, Madden TL, Schäffer AA, et al.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* (1997); 25(17):3389–402.

[31] **Livak KJ, Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001); 25(4):402–8.

[32] **Danby CS, Boikov D, Rautemaa-Richardson R, et al.** Effect of pH on in vitro susceptibility of *Candida glabrata* and *Candida albicans* to 11 antifungal agents and implications for clinical use. *Antimicrob Agents Chemother* (2012); 56(3):1403–6.

[33] **Schmidt P, Walker J, Selway L, et al.** Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*. *Proteomics* (2008); 8(3):534–44.

[34] **Richter SS, Galask RP, Messer SA, et al.** Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol* (2005); 43(5):2155–62.

[35] **Nobile CJ, Fox EP, Nett JE, et al.** A recently evolved transcriptional network controls biofilm development in *Candida albicans*. *Cell* (2012); 148(1–2):126–38.

[36] **Nosanchuk JD, Nimrichter L, Casadevall A, et al.** A role for vesicular transport of macromolecules across cell walls in fungal pathogenesis. *Commun Integr Biol* (2008); 1(1):37–9.

[37] **Nickel W, Rabouille C**. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* (2009); 10(2):148–55.

[38] **Iraqui I, Garcia-Sanchez S, Aubert S, et al.** The Yak1p kinase controls expression of adhesins and biofilm formation in *Candida glabrata* in a Sir4p-dependent pathway. *Mol Microbiol* (2005); 55(4):1259–71.

[39] **de Groot PWJ, Kraneveld EA, Yin QY, et al.** The cell wall of the human pathogen *Candida glabrata* : differential incorporation of novel adhesin-like wall proteins. *Eukaryot Cell* (2008); 7(11):1951–64.

[40] **Bairwa G, Kaur R**. A novel role for a glycosylphosphatidylinositol-anchored aspartyl protease, CgYps1, in the regulation of pH homeostasis in *Candida glabrata. Mol Microbiol* (2011); 79(4):900–13.

[41] **Chaffin WL, López-Ribot JL, Casanova M, et al.** Cell wall and secreted proteins of *Candida albicans:* identification, function, and expression. *Microbiol Mol Biol Rev* (1998); 62(1):130–80.

Chapter 4

Hormones influence on biofilm formation and resistance of *Candida* vaginal isolates

Main goal

To study the influence of progesterone and β-estradiol, at normal cycle and pregnancy concentrations, on biofilm formation and susceptibility to azoles and hydrogen peroxide, of *Candida albicans* and *Candida glabrata* vaginal isolates.

Conclusions

Hormones showed a species-specific modulation of *Candida* biofilm formation and were found to increase cells susceptibility to hydrogen peroxide and azoles, what may have implications in *Candida* vaginal pathogenicity and treatment of VVC.

This chapter is based on the following article:

Gonçalves B, Azevedo NM, Henriques M, Silva S. Hormones modulate *Candida* vaginal isolates biofilm formation and decrease their susceptibility to azoles and hydrogen peroxide. *Medical Mycology* (2019). Doi: 10.1093/mmy/myz070

4.1 Introduction

Vulvovaginal candidiasis (VVC) is a common mucosal infection caused by *Candida* species, mainly *Candida albicans* and *Candida glabrata* [1,2]. *Candida* organisms are opportunistic fungi, whose proliferation is limited by vaginal defences, including hydrogen peroxide liberated by lactobacilli [3,4]. Hydrogen peroxide causes oxidative stress by the formation of hydroxyl radicals, that attack membrane lipids, DNA and other essential fungal cell components [5]. *Candida glabrata* is inherently less susceptible, than *C. albicans,* to hydrogen peroxide [6], and to the most commonly prescribed antifungal agents, as fluconazole [7,8]. Indeed, fluconazole is a first-generation antifungal agent that has been shown to be effective in the treatment of the majority of uncomplicated VVC episodes caused by *C. albicans*, but it is poorly effective in *C. glabrata* infections [8,9]. It has been suggested that voriconazole, which is approved for invasive candidiasis [10], could emerge as new alternative for the treatment of VVC caused by fluconazole-resistant strains [11], minimizing the prolonged use of poorly effective therapy that contribute to the selection of highly resistant species [12]. Voriconazole is a second-generation triazole, structurally related with fluconazole, that presents higher toxicity to the host [10], but also higher activity and spectrum, including fluconazole-resistant species as *C. glabrata* [13,14].

In healthy women, the development of VVC is often triggered by alterations in the vaginal environment that lead to increased hormonal concentration, as pregnancy and use of oral contraceptives [1,15]. Additionally, in the absence of known risk factors, VVC most often occurs in the luteal phase of the menstrual cycle, when progesterone and β-estradiol secretion is elevated [16]. It has been proposed that reproductive hormones contribute to VVC by affecting the host, fungal cells or both. Hormones may affect the host by stimulating the glycogen liberation in the vaginal environment [17] and inhibiting the immune response [18]. Additionally, *Candida* cells have cytosolic receptors that display high affinity for estradiol and progesterone (estrogen- and corticosteroid-binding proteins, respectively) [19,20]. However, the effect of hormones on *Candida* virulence is not entirely clear. It has been shown that progesterone [21–23] and estradiol [24–26] may stimulate, inhibit or do not affect *C. albicans* growth and/or filamentous forms formation, depending on strain and experimental conditions. Moreover, hormones have been shown to induce *C. albicans* expression of several stress-related genes, suggesting lower susceptibility to azoles and oxidative stress [23,25,27].

Although some studies have investigated the effects of progesterone or β-estradiol on *Candida* cells, their influence on *Candida* virulence features is still poorly studied, especially in *C. glabrata* species. Therefore, this work aimed to evaluate the effect of progesterone and β-estradiol, at normal

menstrual cycle and pregnancy levels, on *C. albicans* and *C. glabrata* planktonic growth, biofilm formation, filamentous forms development (*C. albicans*) and susceptibility to azoles and hydrogen peroxide. The objective of this work was to deepen the current knowledge about the influence of reproductive hormones on *C. albicans* and *C. glabrata* virulence and resistance, contributing to a better understanding of the pathogenesis of these species in the vaginal environment.

4.2 Materials and Methods

4.2.1 Strains and initial culture conditions

In this work two strains of *Candida* were used, namely *C. albicans* 569322 and *C. glabrata* 534784. These strains are vaginal isolates belonging to the Biofilm group collection, located at the Centre of Biological Engineering of Minho University (Braga, Portugal), and were previously isolated from patients of Hospital of S. Marcos, Braga, Portugal. To confirm the identity of the isolates, CHROMagar *Candida* (CHROMagar, Paris, France) and PCR-based sequencing with specific primers (ITS1 and ITS4) were used [28].

The strains used in this study were grown in Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h, followed by 18 h in Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany) as described in Chapter 2- section 2.2.1. For the subsequent planktonic growth or biofilm development, the cells were suspended in Roswell Park Memorial Institute (RPMI; Sigma-Aldrich, St Louis, MO, USA) medium adjusted to pH 4 (with lactic acid) and the cellular density was estimated using a Neubauer haemocytometer for cells counting. The experiments described in next sections were performed in triplicate and in three independent assays (pre-inoculums independently prepared).

4.2.2 Hormones

Two hormones were used in this work, progesterone and β-estradiol (Sigma-Aldrich, St Louis, MO, USA). "Stock solutions" of each hormone at 10 mM and "work solutions" at 5000mg/l were prepared on ultrapure water and stored at -20 °C to be used in all experiments. From the "work solutions", several solutions of progesterone and β-estradiol, where prepared in RPMI, at pH 4, at concentrations corresponding to the peaks of normal menstrual cycle (7.5 μg/l and 700 ng/l, respectively) and third trimester of pregnancy (200 μg/l and 15 μg/l, respectively) [29]. In addition, solutions with the combination of both hormones were prepared, at concentrations of the same hormonal status. These solutions were freshly made to use in each experiment.

4.2.3 Planktonic growth analysis

Regarding planktonic growth, cellular suspensions of *C. albicans* and *C. glabrata* strains were prepared at $1x10^5$ cells/ml in the hormone-containing solutions described above (6 conditions). A control suspension, of each strain, without hormones, was also prepared (RPMI at pH 4). All cellular suspensions were placed in 25 ml Erlenmeyer flasks and incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev/min). After incubation, planktonic cells were analysed or used in the susceptibility assays.

Planktonic cells were analysed in terms of cultivability through the colony forming units (CFU) counting methodology [30] by plating on SDA a serial of 10-fold dilutions of the planktonic suspensions and counting the number of CFUs after 24 h of incubation at 37°C. The results were presented as Log CFUs/ml.

Additionally, *C. albicans* planktonic cells were also evaluated in terms of filamentous forms formation. For that, aliquots of each cellular suspension were diluted in sterile water (1:100) and the percentage of filamentous forms was counted in an optical microscope using a Neubauer chamber.

4.2.4 Biofilm formation and analysis

In order to develop *C. albicans* and *C. glabrata* biofilms, cellular suspensions with $1x10^5$ cells/ml were prepared in RPMI (pH 4) containing progesterone and/or β-estradiol, at the concentrations previously described (6 conditions). A hormone-free suspension of each strain was also prepared (RPMI at pH 4). Then, all suspensions were placed into wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) (200 µl per well). The plates were incubated at 37°C under agitation (120 rev/min) for 24 h [30]. The medium was aspirated and biofilms were washed with Phosphate Buffered Saline (PBS) twice. The biofilms were analysed directly or their cells were removed to perform the susceptibility test assays.

4.2.4.1 Biomass and biofilm cells' cultivability analysis

The biofilms' total biomass was estimated by Crystal Violet (CV) staining methodology [30], as described in Chapter 2 - section 2.2.4. The results were presented per unit area of biofilm (Absorbance CV/cm^2). The number of cultivable cells in the biofilms was determined by CFU counting methodology

[31], as described in Chapter 2 - section 2.2.5. The results were presented per unit area of biofilm (Log (CFUs/ml)/cm^2).

4.2.4.2 Filamentous forms analysis

In order to analyse the formation of filamentous forms in *C. albicans* biofilm cells, biofilms were scraped from the microtiter plate wells with PBS and the suspensions were vortexed for 2 min to disaggregate the cells [30]. Then, aliquots of these suspensions were used to determine the percentage of filamentous forms using a Neubauer chamber. In parallel, the morphology of cells was confirmed through epifluorescence microscopy (Olympus BX51 coupled with a DP71 digital camera; Olympus, Tokyo, Japan), after staining the cells with 1% (v/v) of Calcofluor white for 10 min.

4.2.5 Susceptibility tests

To perform the susceptibility tests to azoles and hydrogen peroxide, *C. albicans* and *C. glabrata* strains were grown in the planktonic and biofilm lifestyles during 24 h, in the absence and presence of hormones, as described above. To recover the biofilm cells, biofilms were scraped from the wells with PBS and the suspensions were vortexed for 2 min [30]. The biofilm cells-containing suspensions and the suspensions of cells grown planktonically were all centrifuged at 5000 g for 5 min at 4 °C. Pellets were used to prepare cellular suspensions in RPMI at pH 4, supplemented with the same hormones' concentrations used in the previous planktonic/biofilm growth. These suspensions with $2.5x10^3$ cells/ml were prepared using a Neubauer haemocytometer for cells counting, and then used for the susceptibility tests to azoles and hydrogen peroxide.

4.2.5.1 Azoles testing

Two antifungal agents, fluconazole and voriconazole (Pfizer, New York, NY, USA), were tested. Stock solutions of these agents were prepared according to Clinical and Laboratory Standards Institute (CLSI) guidelines, using the appropriate solvents, dimethyl-sulfoxide (DMSO) and water [32]. Stock solutions were divided into aliquots and stored in vials at -20 °C. For each experiment, serial dilutions of antifungal solutions were prepared in RPMI at pH 4, to be used in the susceptibility tests.

The cellular suspensions with $2.5x10^3$ cells/ml, prepared in the presence and absence of hormones, were used to perform the susceptibility assays according to the CLSI M27 reference protocol for broth microdilution antifungal susceptibility testing of yeasts [32]. It should be noted that CLSI reference method does not include the previous planktonic or biofilm growth performed in this study, what was adapted by adjusting the initial cellular concentration to that indicated in the reference protocol ($2.5x10^3$ cells/ml). Preliminary susceptibility tests with *C. albicans* strain to fluconazole and *C. glabrata* strain to fluconazole and voriconazole, in the absence of hormones, were performed to define the suitable concentrations range to be used in the tests in the presence of hormones. Due to the high resistance of *C. glabrata* strain to fluconazole, the susceptibility tests against this agent were not performed. In all assays, the Minimum Inhibitory Concentrations (MICs) were determined visually and spectrophotometrically, according to CLSI guidelines [32].

4.2.5.2 Hydrogen peroxide testing

To test the susceptibility of *Candida* strains to hydrogen peroxide, the CLSI M27 protocol [32] was adapted, using different concentrations of hydrogen peroxide (100 to 300 mg/l in *C. albicans* and 800 to 1500 mg/l in *C. glabrata* strain). Solution were prepared in RPMI at pH 4 with a stock solution of Hydrogen Peroxide 30 % w/w in water (Sigma-Aldrich, St Louis, MO, USA). Similar to antifungals testing, hormone-containing suspensions of planktonic and biofilm cells ($2.5x10^3$ cells/ml) were used, and MICs determination was performed according to CLSI protocol [32]. The Minimum Fungicidal Concentration (MFC) was also determined by plating on SDA the cellular suspensions obtained in the susceptibility tests to which the peroxide hydrogen concentrations led to no visible growth. The SDA plates were incubated for 24 h at 37°C and the presence/absence of colonies was visualized.

4.2.6 Statistical analysis

The results of cells density, cultivability, biomass and filamentous forms percentage were statistically analysed using GraphPad Prism 6 software. For that, the results obtained for each strain in the presence of hormones were compared with those obtained in their absence (control) using one-way ANOVA and Dunnett's multiple comparisons test. Additionally, the comparison between *C. albicans* and *C. glabrata* cells cultivability and between biofilm and planktonic cells filamentation, was performed for each condition, using the t test analysis. All tests were performed with a confidence level of 95%.

4.3 Results

4.3.1 Hormones influence on *Candida* planktonic growth

In order to determine the influence of hormones on planktonic growth of *C. albicans* and *C. glabrata* vaginal isolates, free-floating cells were incubated for 24 h in the absence and presence of progesterone and/or β-estradiol, at normal cycle and pregnancy concentrations. The planktonic growth was evaluated in terms of cultivability, through CFU determination. There were no statistical differences (P-value >0.05) between the absence and presence of hormones, in both species (Figure 4.1). Additionally, *C. glabrata* presented statistically (P-value ≤0.05) higher number of cultivable cells than *C. albicans*, of approximately 1 order of magnitude (Log CFU/ml), in unsupplemented or supplemented conditions (Figure 4.1).

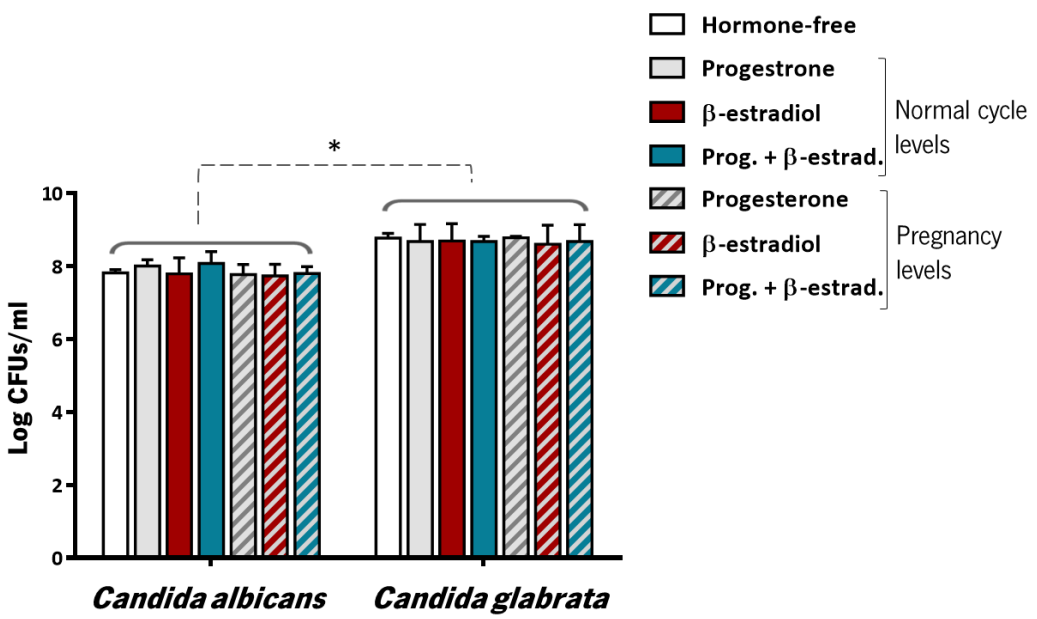


Figure 4.1 Effect of hormones on *Candida albicans* and *Candida glabrata* planktonic growth. Cultivable cells determination (Log CFUs/ml) of *C. albicans* 569322 and *C. glabrata* 534784 strains grown planktonically during 24 h in RPMI at pH 4, in the absence (hormone-free) and presence of progesterone and/or β-estradiol, at concentrations corresponding to the normal menstrual cycle (7.5 μg/l and/or 700 ng/l, respectively) and pregnancy (200 μg/l and/or 15 μg/l, respectively). Error bars represent standard deviation. Asterisk represent statistical difference of *C. glabrata* results in comparison with those of *C. albicans* (P-value ≤0.05), in all conditions.

4.3.2 Hormones modulation of *Candida* biofilm formation

In order to determine the influence of hormones on biofilm formation ability of *Candida* vaginal isolates, biofilms were performed during 24 h in the absence and presence of progesterone and/or β-estradiol, at normal cycle and pregnancy concentrations, as described in the material and methods section. Formed biofilms were analysed in terms of cultivable cells (Figure 4.2-A) and total biomass (Figure 4.2-B).

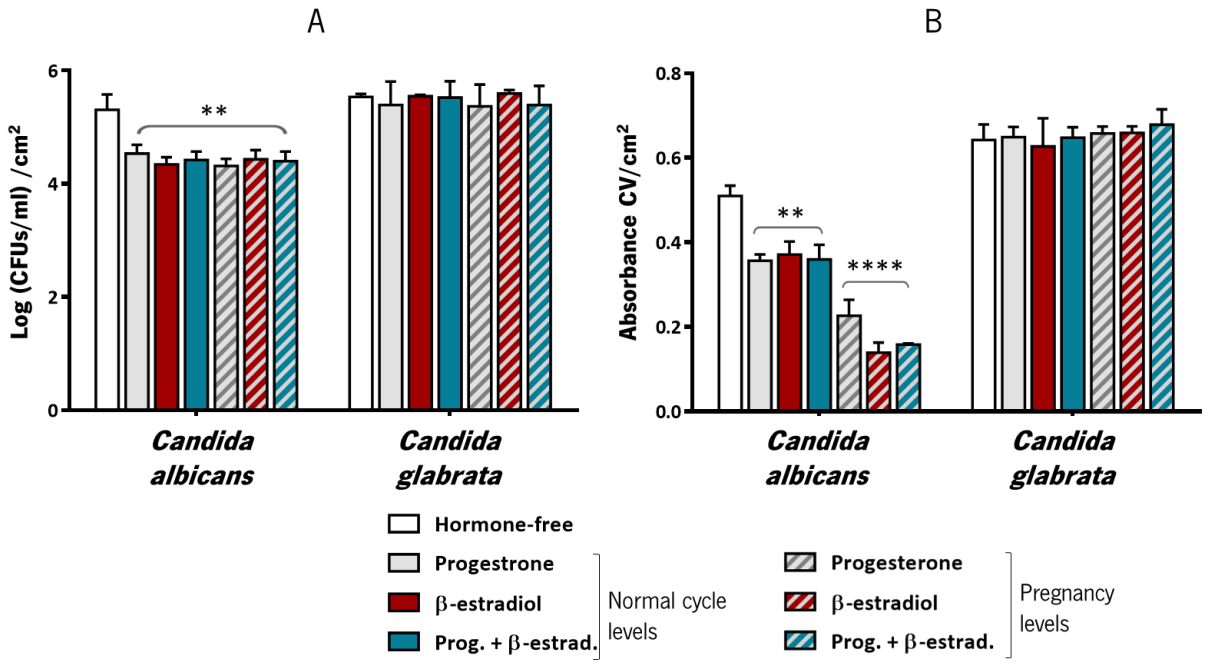


Figure 4.2 Effect of hormones on *Candida albicans* and *Candida glabrata* biofilm formation. (A) Cultivable cells determination (Log (CFUs/ml)/cm²) and **(B)** total biomass quantification (Absorbance CV/cm²) of *C. albicans* 569322 and *C. glabrata* 534784 biofilms developed during 24 h in RPMI at pH 4, in the absence (hormone-free) and presence of progesterone and/or β-estradiol, at concentrations corresponding to the normal menstrual cycle (7.5 µg/l and/or 700 ng/l, respectively) and pregnancy (200 µg/l and/or 15 µg/l, respectively). Error bars represent standard deviation. Asterisks represent statistical differences when compared with the respective hormone-free control (**** P-value ≤0.0001; ** P-value ≤0.01).

The hormones had no significant effect (P-value >0.05) on *C. glabrata* biofilm formation, in terms of cultivable cells or total biomass, at all concentrations tested (Figure 4.2-A,B). In contrast, *C. albicans* biofilms formed in the presence of hormones had statistically lower (P-value ≤0.01) biomass and number of cultivable cells, than those developed in the hormone-free environment (Figure 4.2-A,B). Biofilm cultivable cells decreased approximately 1 order of magnitude (Log (CFUs/ml)/cm^2), independently of the hormone(s) concentration tested (Figure 4.2-A). Differentially, biofilm total biomass presented a dose-dependent decrease, of about 30 and 65%, in the presence of hormones at normal cycle and pregnancy concentrations, respectively. Additionally, there were no statistical differences (P-value >0.05) between individual and mutual hormonal supplementation, at concentrations corresponding to the same hormonal status (Figure 4.2-B).

4.3.3 Hormones effect on *C. albicans* filamentous forms development

Cells of *C. albicans* 569322 grown in planktonic and biofilm lifestyles during 24 h were analysed by optical microscopy in order to evaluate the hormones effect on filamentous forms development (Figure 4.3). In a hormone-free environment, biofilms presented higher percentage of cells in filamentous form than planktonic ones (P-value ≤0.01) (Figure 4.3-A). Moreover, the filamentation of planktonic cells was not significantly (P-value >0.05) affected by the hormones. Differentially, filamentation of biofilm cells in the presence of hormones presented a statistically significant decrease (P-value ≤0.001) comparatively to the hormone-free environment (Figure 4.3-A). There was a trend to a higher impact in the presence of the highest hormones concentrations, being the lowest reduction, of 46%, caused by progesterone at normal cycle concentrations, and the highest, of 80%, by mutual supplementation (progesterone and β-estradiol) at pregnancy levels.

Biofilm cells grown the in presence of the highest hormonal concentrations (pregnancy) were stained with calcofluor white and observed by epifluorescence microscopy (Figure 4.3-B), in order to confirm the results obtained in Figure 4.3-A. The images corroborate the microbiological studies, demonstrating not only a reduction in the number of filaments but also in their length. The effect on filamentous length was similar with hormones supplemented individually and conjugated.

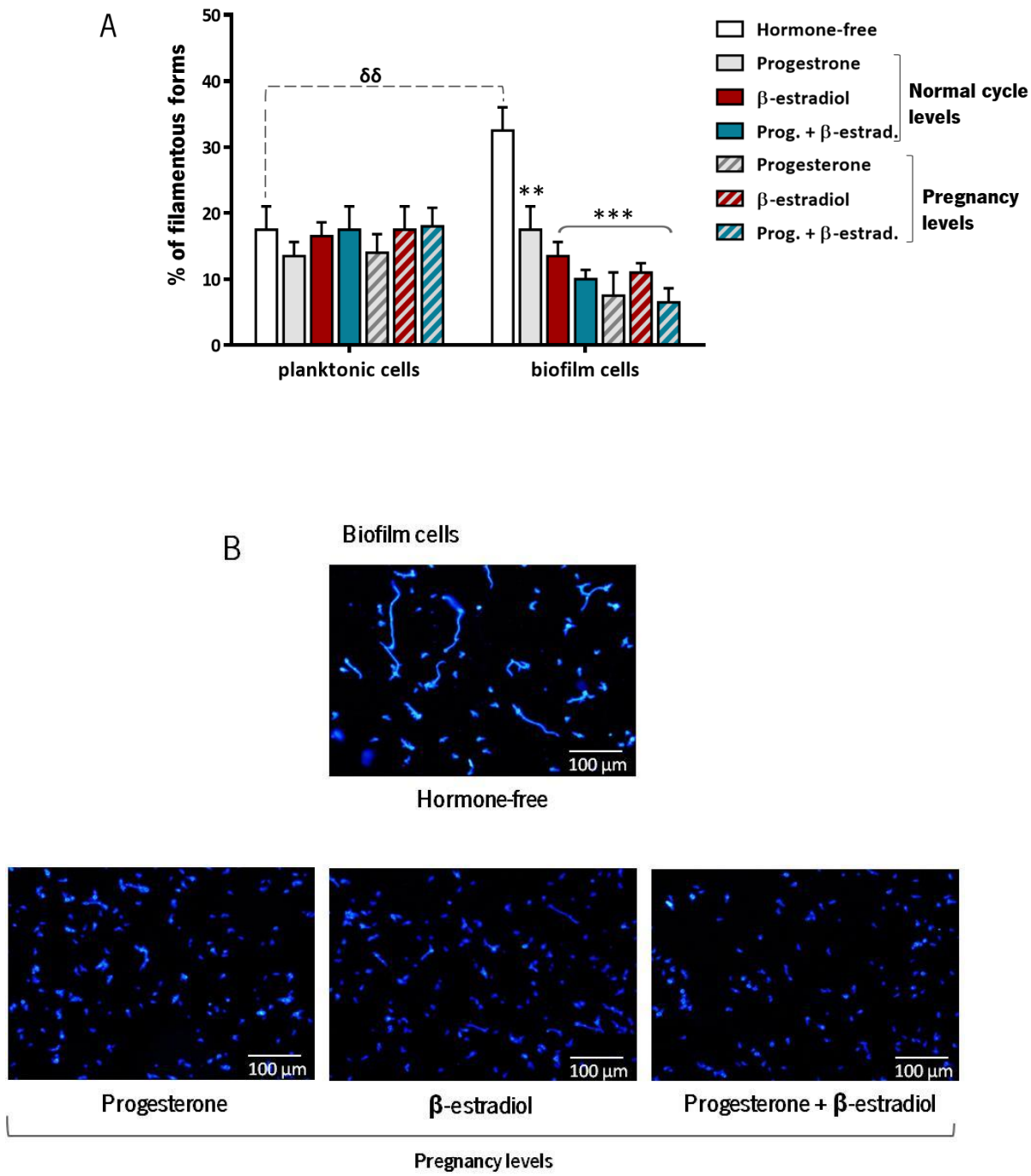


Figure 4.3 Effect of hormones on *Candida albicans* filamentous forms development. (A) Filamentous forms percentage of *C. albicans* 569322 planktonic and biofilm cells grown for 24 h in RPMI at pH 4, in the absence (hormone-free) and presence of progesterone and/or β-estradiol at concentrations corresponding to the normal menstrual cycle (7.5 µg/l and/or 700 ng/l, respectively) and pregnancy (200 µg/l and/or 15 µg/l, respectively). Error bars represent standard deviation. Asterisks represent statistical differences when compared with the respective hormone-free control (*** P-value ≤0.001; ** P-value ≤0.01). Deltas represent statistical difference between the results of planktonic and biofilm cells in hormone-free conditions (δδ P-value ≤0.01). **(B)** Images of *C. albicans* 569322 biofilm cells, grown in hormone-free environment and in the presence of progesterone, β-estradiol or both hormones, at pregnancy levels. Images were obtained in an epifluorescence microscope (Olympus BX5), coupled with a DP71 digital camera. Original magnification was 10x and the scale bars correspond to 100 µm.

4.3.4 Hormones influence on *Candida* cells resistance

The influence of hormones, on the susceptibility of *C. albicans* 569322and *C. glabrata* 534784 cells to azoles (fluconazole and voriconazole, respectively) and hydrogen peroxide was evaluated using CLSI (M27) methodology. Importantly, cells were previously grown in planktonic and biofilm lifestyles during 24 h in similar hormonal conditions. Table 4.1 presents the results of the minimum concentrations of fluconazole and voriconazole that inhibit 50% (MIC_{50}) or 90% (MIC_{90}) of *C. albicans* and *C. glabrata* cells growth, respectively. The susceptibility assays for *C. glabrata* vaginal isolate were performed with voriconazole, since in the preliminary susceptibility assays *C. glabrata* 534784 was highly resistant to fluconazole (MIC_{50} >64mg/l). Table 4.2 presents the results of MIC_{50}, MIC_{90} and minimum fungicidal concentrations (MFC) of hydrogen peroxide, for both strains.

4.3.4.1 Susceptibility to azoles

The hormones led to a dose-dependent decrease of *C. albicans* and *C. glabrata* susceptibility to fluconazole and voriconazole, respectively (Table 4.1). Hormones, at normal cycle and pregnancy concentrations, increased *C. albicans* fluconazole MIC_{50} up to 2 and 11 times, and *C. glabrata* voriconazole MIC_{50} up to 1.5 and 6 times, respectively, in relation to the absence of hormones. The results of MIC_{90} show a similar trend, but the impact was consistently greater on MIC_{50}, for both species (Table 4.1). In *C. glabrata*, the presence of both hormones (at pregnancy levels) had higher impact on MICs than individual supplementation. Differentially, *C. albicans* presented similar impact in the presence of β-estradiol and both hormones, that was higher than in the presence of progesterone. Additionally, there was not a relevant susceptibility difference between planktonic and biofilm cells, although the results point to a slightly lower susceptibility of biofilm cells to azoles (Table 4.1).

Table 4.1 Susceptibility of *Candida albicans* and *Candida glabrata* to fluconazole and voriconazole in the presence of progesterone and/or β-estradiol. Minimum inhibitory (MIC) concentrations (mg/l) of fluconazole and voriconazole for *C. albicans* 569322 and *C. glabrata* 534784 cell growth, respectively. The susceptibility tests were performed with RPMI at pH 4 in the absence (control) or presence of progesterone and/or β-estradiol, at normal menstrual cycle (luteal phase) and pregnancy (3th trimester) concentrations. Tested cells were previously grown in planktonic or biofilm lifestyle during 24 h in RPMI at pH 4, in similar hormonal conditions. The most relevant results are in bold.

Hormones	**Previous lifestyle**	***Candida albicans*** Fluconazole		***Candida glabrata*** Voriconazole	
		MIC_{50}	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**
Control (hormone-free)	Planktonic	0.2	0.5	2	10
	Biofilm	0.3	0.6	2	10
Progesterone (normal cycle)	Planktonic	0.4	0.75	2.5	10
	Biofilm	0.4	0.75	2.5	11
β-estradiol (normal cycle)	Planktonic	0.3	0.6	2.5	10
	Biofilm	0.4	0.75	3.5	11
Progesterone + β-estradiol (normal cycle)	Planktonic	0.4	0.6	2.5	10
	Biofilm	0.4	0.75	3.5	11
Progesterone (pregnancy)	Planktonic	**0.7**	**1**	**10**	**25**
	Biofilm	**0.75**	**2**	**11**	**25**
β-estradiol (pregnancy)	Planktonic	**2.4**	**2.75**	**10**	**25**
	Biofilm	**2.5**	**3**	**11**	**25**
Progesterone + β-estradiol (pregnancy)	Planktonic	**2.5**	**3**	**12**	**30**
	Biofilm	**2.5**	**3**	**16**	**30**

MIC_{50} – Minimum inhibitory concentration (mg/l) of 50% of cell growth
MIC_{90} – Minimum inhibitory concentration (mg/l) of 90% of cell growth

4.3.4.2 Susceptibility to hydrogen peroxide

The hormones decreased the susceptibility of *C. albicans* and *C. glabrata* vaginal isolates to hydrogen peroxide, in a dose-dependent manner (Table 4.2), although the dependency was not as evident as in antifungal susceptibility. In a hormone-free environment, *C. glabrata* presented intrinsically higher resistance to hydrogen peroxide than *C. albicans* (MIC_{50} of 850-875 and 155-175 mg/l, respectively). In both species, the presence of hormones increased the hydrogen peroxide MIC_{50} up to 10 and 22%, with normal cycle and pregnancy concentrations, respectively (Table 4.2). The results of

MIC_{90} and MFC show a similar trend, although in *C. glabrata* it was not possible to evaluate the impact on MFC, as it was higher than the highest concentration tested (1500 mg/l), in all conditions. Moreover, the presence of progesterone, β-estradiol and mutual supplementation had similar impact, in both species. Additionally, similarly to azoles susceptibility, cells previously grown in planktonic lifestyle did not show relevant susceptibility differences from those previously grown in the biofilm lifestyle, although the results suggest lower susceptibility of biofilm cells to hydrogen peroxide (Table 4.2).

Table 4.2 Susceptibility of *Candida albicans* and *Candida glabrata* to hydrogen peroxide in the presence of progesterone and/or β-estradiol. Minimum inhibitory (MIC) and fungicidal (MFC) concentrations (mg/l) of hydrogen peroxide for *C. albicans* 569322 and *C. glabrata* 534784 cell growth. The susceptibility tests were performed with RPMI at pH 4 in the absence (control) or presence of progesterone and/or β-estradiol, at normal menstrual cycle (luteal phase) and pregnancy (3th trimester) concentrations. Tested cells were previously grown in planktonic or biofilm lifestyle during 24 h in RPMI at pH 4, in similar hormonal conditions.

Hormones	Previous lifestyle	*Candida albicans*			*Candida glabrata*		
		MIC_{50}	**MIC_{90}**	**MFC**	**MIC_{50}**	**MIC_{90}**	**MFC**
Control (hormone-free)	Planktonic	155	180	210	875	900	>1500
	Biofilm	175	190	210	850	900	>1500
Progesterone (normal cycle)	Planktonic	175	210	220	950	975	>1500
	Biofilm	205	215	220	950	975	>1500
β-estradiol (normal cycle)	Planktonic	180	210	225	950	1000	>1500
	Biofilm	210	210	225	975	1000	>1500
Progesterone + β-estradiol (normal cycle)	Planktonic	180	210	225	950	1000	>1500
	Biofilm	210	215	225	975	1000	>1500
Progesterone (pregnancy)	Planktonic	190	220	240	1000	1250	>1500
	Biofilm	215	225	240	1100	1250	>1500
β-estradiol (pregnancy)	Planktonic	195	220	240	1100	1250	>1500
	Biofilm	225	220	240	1100	1250	>1500
Progesterone + β-estradiol (pregnancy)	Planktonic	200	225	240	1100	1250	>1500
	Biofilm	225	225	240	1100	1250	>1500

MIC_{50} – Minimum inhibitory concentration (mg/l) of 50% of cell growth
MIC_{90} – Minimum inhibitory concentration (mg/l) of 90% of cell growth
MFC – Minimum fungicidal concentration (mg/l)

4.4 Discussion

Women hormonal status has been shown to be relevant for VVC development, as conditions leading to high hormonal levels, including pregnancy, are considered risk factors to the establishment of the disease [1,15]. However, the effect of hormones on *Candida* cells is still poorly studied, being mostly limited to the influence of progesterone or estradiol on *C. albicans* strains, in which has been reported high dependence on strain and environmental conditions [22,23,25,26]. In this work, vaginal isolates of *C. albicans* and *C. glabrata* were used, and the growth medium (RPMI) was adjusted to pH 4 with lactic acid, in order to mimic the vaginal acidity (pH in range of 3.6-4.5). VVC often occurs in the menstrual phase with the highest hormones secretion [16] and is increased in pregnancy [15], thus, progesterone and β-estradiol were used at maximal peaks of normal menstrual cycle (7.5 μg/l and 700 ng/l, respectively) and third trimester of pregnancy (200 μg/l and 15 μg/l, respectively) [29]. As such, it was aimed to study the potential contribution of hormones to *Candida* vaginal pathogenicity, mainly to its ability to develop biofilms and susceptibility to biological and non-biological harmful substances.

The presence of hormones on *C. albicans* and *C. glabrata* planktonic growth did not reveal a relevant effect, at any concentration tested (Figure 4.1), in accordance with some previous reports [22,25,26]. Interestingly, *C. glabrata* presented higher number of planktonic cultivable cells than *C. albicans*, in all conditions (Figure 4.1), and its biofilm formation was not affected by the presence of hormones, (Figure 4.2), suggesting high adaptation of *C. glabrata* to vaginal conditions. Previous reports have shown that *C. glabrata* cells can adapt to different stress conditions, including low pH [33,34]. The high adaptation ability of *C. glabrata* cells to specific vaginal conditions may contribute to its intrinsically high resistance to host defences and antifungal agents commonly used to treat VVC [7]. Additionally, *C. glabrata* strains have been commonly isolated among patients suffering from recurrent episodes of VVC [35,36], which have a potential relation with biofilm formation, as biofilms may hamper the complete eradication of the pathogen from the vaginal lumen, leading to recurrence [37].

Contrarily to *C. glabrata*, *C. albicans* biofilm formation was affected by the presence of progesterone and/or β-estradiol, at all concentrations tested (Figure 4.2). Alves et al. [22] reported a similar result concerning *C. albicans* biofilm formation ability in the presence of progesterone. Interestingly, the reduction of biofilm cultivable cells was similar in all concentrations tested (Figure 4.2-A), but the decrease of biofilm total biomass was dose-dependent (Figure 4.2-B). This result suggests that, in the presence of hormones, *C. albicans* biofilms present not only lower number of cells but also altered morphologic features that affect the biomass results. In fact, we found that hormones decreased *C. albicans* biofilm cells ability to produce filamentous forms, in terms of quantity and length (Figure

4.3-A.B). As expected, in the absence of hormones, biofilm cells presented more filaments than planktonic cells (Figure 4.3-A), due to the induction of filamentous forms formation in the biofilm lifestyle [38]. Interestingly, filamentous forms formation ability of planktonic cells was not significantly affected by hormones, showing a specific effect on biofilm cells (Figure 4.3-A). Previous studies have shown that the presence of progesterone or β-estradiol influences *C. albicans* filamentous forms development, being reported stimulation [25,39], reduction [21,26] or none effect [25], depending on strain and environmental conditions. Additionally, we found that the conjugation of progesterone and β-estradiol did not appear to have significantly higher effect than of only one hormone (at levels of the same hormonal status) and was usually limited to the maximum observed in the individual supplementation.

It has been shown that some environmental conditions, including low pH, induce the dispersion of mature *C. albicans* biofilms, a key step of the biofilm development cycle, by decreasing the filamentation and adhesion of mature biofilm cells [40]. As in this study were found similar effects on *C. albicans* biofilm cells in the presence of hormones, it may be speculated that hormones may also induce *C. albicans* biofilm dispersion, contributing to the establishment of new foci of infection. The biofilm dispersion ability is relevant in the vaginal environment, especially in women using intrauterine devices, to which *Candida* species can easily adhere and form biofilms, being a source of highly virulent pathogens [41]. Importantly, biofilm dispersed cells have been shown to display potentiated virulence traits compared to their planktonic counterparts, including enhanced adhesion, filamentation and biofilm formation abilities [40].

In order to get further insights into the hormones' effects on *C. albicans* and *C. glabrata* cells, their influence on cells' susceptibility to the antifungals, fluconazole and voriconazole, respectively, was also evaluated. These agents are first- and second-generation triazoles, respectively, and exert their activity through the inhibition of ergosterol biosynthesis [42,43]. The *C. albicans* strain used in this study presented MIC_{50} and MIC_{90} of 0.2-0.6 mg/l (Table 4.1), in the absence of hormones, being categorized as susceptible to fluconazole, according to CLSI interpretive breakpoints (MIC ≤2mg/l) [44]. In the presence of progesterone and/or β-estradiol it was observed a dose-dependent decrease of cells susceptibility (Table 4.1). The presence of normal cycle hormone concentrations increased *C. albicans* fluconazole MICs up to 0.75 mg/l, thus remaining in the susceptible phenotype, according to CLSI [44]. However, in the presence of hormones at pregnancy concentrations, MICs increased up to 3 mg/l (Table 4.1), exceeding the upper bound of susceptible categorization of 2 mg/l [44]. Similarly, Banerjee et al. [23] found higher MIC of fluconazole in *C. albicans* cells exposed to supra-physiologic

concentration of progesterone, than in its absence (MIC_{80} of 2 and 0.5 mg/l, respectively). In accordance, higher expression of efflux pumps (*CDR1* and *CDR2*) have been found in *C. albicans* cells exposed to hormones, which are thought to pump them out of cells, minimizing their effect on expression of other genes [23,25,45]. Our results suggest that the treatment of susceptible *C. albicans* strains with fluconazole may present higher difficulty in the presence of high hormonal levels, potentially leading to antifungal therapy failure. It should be noted that the comparison of our results with CLSI breakpoints has some limitations, as pH 4 and cells in a growth phase different from CLSI protocol were used, which could enhance the antifungal susceptibility of cells [33,46]. In fact, an acidic pH has been shown to increase *C. albicans* and *C. glabrata* susceptibility to different antifungal agents, suggesting that in the selection of agents for VVC treatment, clinicians should recognize the limitations of *in vitro* susceptibility testing at neutral pH [33,47].

Contrarily to *C. albicans*, the *C. glabrata* vaginal isolate (534784) is highly resistant to fluconazole (MIC_{50}≥ 64 mg/l) (data not shown), suggesting that this agent would probably be ineffective in a vaginal infection of this isolate. Thus, for this species voriconazole was used instead of fluconazole to perform the susceptibility assays, as voriconazole has been shown to be effective against fluconazole-resistant strains [11,14]. In the absence of hormones, the *C. glabrata* strain tested presented a voriconazole MIC_{50} of 2 mg/l (Table 4.1). CLSI does not have defined breakpoints of *C. glabrata* susceptibility to voriconazole, because there is not yet data available to indicate correlation between MIC and treatment outcome. However, it is reported that the majority of isolates exhibit MIC of voriconazole of less than 1 mg/l and fluconazole-resistant isolates usually present higher MIC [48], in accordance to our results. In the presence of progesterone and/or β-estradiol, *C. glabrata* susceptibility was decreased in a dose-dependent manner, with a slightly higher impact in the presence of both hormones, comparatively to individual supplementation (Table 4.1). At low concentrations of hormones the MIC_{50} of voriconazole was 2.5-3.5 mg/l, however it increased up to 12-16 mg/l at pregnancy concentrations, a level much higher than achievable in serum with dosing regimens at the limit of voriconazole toxicity (5.5mg/l) [10]. Our results suggest limited therapeutic efficiency of voriconazole in the presence of high hormonal levels. Importantly, although fluconazole and voriconazole are usually considered to be fungistatic, it has been demonstrated that at high concentrations they could also have fungicidal activity in *Candida* species, although this effect seems to be strain dependent [49,50]. In this work it was not found fungicidal effect of fluconazole or voriconazole on *C. albicans* and *C. glabrata* strains (or even 100% of growth inhibition), respectively, tested up to 70 mg/l, in all conditions. As such, the potential fungicidal activity of these agents was not achieved in their range of safe toxicity (up to 70 mg/l in

fluconazole and 5.5 mg/l in voriconazole), what has been suggested to be a relevant clinical outcome to immunocompromised hosts, where inhibition of *Candida* growth may not be sufficient [10,51].

Besides azoles susceptibility, the influence of hormones on *Candida* susceptibility to hydrogen peroxide was also evaluated. Hydrogen peroxide is released in the vaginal environment by *Lactobacillus* species and is thought to act as biological barrier of VVC development [3,4]. Although, some vaginal substances may impair anti-*Candida* effect of hydrogen peroxide, low pH is suggested to stabilize it [52]. *Candida albicans* and *C. glabrata* presented MICs of hydrogen peroxide, in the absence of hormones (850-900 mg/l and 155-190 mg/l, respectively), near to those previously reported, although indicative of strain or pH influence [5,6,52]. Importantly, fungicidal levels for *C. albicans* were low (MFCs of 210-240 mg/l), whereas in *C. glabrata* were not achieved until 1500 mg/l, in any condition (Table 4.2). The lower susceptibility of *C. glabrata* to hydrogen peroxide is in accordance with a higher number of oxidative stress-related genes reported in *C. glabrata* (64 genes), comparatively to *C. albicans* (49 genes) [53]. In the presence of hormones, *C. albicans* and *C. glabrata* presented decreased susceptibility to peroxide hydrogen (Table 2), in a dose-dependent manner (MIC increase of about 10% with low hormones levels and 22% with pregnancy concentrations). In accordance, previous studies reported increased expression of oxidative stress related genes in the presence of hormones. [23,27]. The impact of progesterone and β-estradiol supplemented individually or together (at the same hormonal status) was similar, in both species. Our results suggest that hormones may contribute to *Candida* survival in the vaginal environment by enhancing its adaptation and response to harmful biological substances.

Unexpectedly, the growth of cells in planktonic or biofilm modes, did not affect their susceptibility to azoles and peroxide hydrogen (in the absence or presence of hormones), although there was a slight trend to lower susceptibility of biofilm cells, in both species (Tables 4.1 and 4.2). It has been reported that biofilms of *C. albicans* and *C. glabrata* have higher expression of efflux pumps (cdr1 and cdr2) and oxidative stress response proteins, which could indicate lower susceptibility of biofilm cells to azoles and peroxide hydrogen [54–56]. Ramage et al. [55] found significantly lower fluconazole susceptibility in *C. albicans* biofilm cells than in planktonic ones, but the biofilm formation procedure was different from ours, and we performed the susceptibility assays with age-matched planktonic and biofilm cells. In fact, Ramage et al. [55] also found that *C. albicans* strains deficient in efflux pumps are hypersensible to fluconazole in planktonic assays but still remain resistant in the biofilm lifestyle. These results suggest, as ours, that the increased resistant phenotype presented by

biofilms is probably mostly potentiated by architectural or community-based mechanisms, not present in planktonic cells, rather than higher individual antifungal resistance of cells within the biofilms.

It is important to point that the use of only one strain of each *Candida* species limits the findings suggested by this study, that have to be confirmed in a greater number of isolates. Additionally, the findings for the azoles' susceptibility should also be confirmed with other azole agents used as primary choice to treat VCC, including clotrimazole, miconazole, econazole and fenticonazole, in order to obtain a broader picture of hormonal influence on VVC treatment.

The results of this study reveal that reproductive hormones are able to module defence and virulence traits of *C. albicans* and *C. glabrata* cells, possibly contributing to their adaptation to the vaginal environment and protecting them against adverse environmental conditions. This study contributes to a better understanding of the influence of reproductive hormones on *Candida* virulence, although further studies are necessary to decipher their complete effect on *Candida* vaginal pathogenicity.

References

[1] **Ahmad A, Khan AU**. Prevalence of *Candida* species and potential risk factors for vulvovaginal candidiasis in Aligarh, India. *Eur J Obstet Gynecol Reprod Biol* (2009); 144(1):68–71.

[2] **Gonçalves B, Ferreira C, Alves CT, et al.** Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. *Crit Rev Microbiol* (2016); 42(6):905–27.

[3] **Boris S, Barbés C**. Role played by lactobacilli in controlling the population of vaginal pathogens. *Microbes Infect* (2000); 2(5):543–6.

[4] **Xu J, Sobel JD**. Antibiotic-associated vulvovaginal candidiasis. *Curr Infect Dis Rep* (2003); 5(6):481–7.

[5] **Orrù G, Del Nero S, Tuveri E, et al.** Evaluation of antimicrobial-antibiofilm activity of a hydrogen peroxide decontaminating system used in dental unit water lines. *Open Dent J* (2010); 4:140–6.

[6] **Qyasian A, Mohammadi Z, Giardino L, et al.** Determining the minimum inhibitory concentration of Tetraclean against *Candida albicans*. *Niger J Med* (2014); 23(3):201–6.

[7] **Richter SS, Galask RP, Messer SA, et al.** Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. *J Clin Microbiol* (2005); 43(5):2155–62.

[8] **Sojakova M, Liptajova D, Borovsky M, et al.** Fluconazole and itraconazole susceptibility of vaginal yeast isolates from Slovakia. *Mycopathologia* (2004); 157(2):163–9.

[9] **Sobel JD, Brooker D, Stein GE, et al.** Single oral dose fluconazole compared with conventional clotrimazole topical therapy of *Candida* vaginitis. *Am J Obstet Gynecol* (1995); 172(4):1263–8.

[10] **EUCAST**. Technical Note on voriconazole. *Clin Microbiol Infect* (2008); 14(10):985–7.

[11] **Gonzalez GM, Portillo OJ, Uscanga GI, et al.** Therapeutic efficacy of voriconazole against a fluconazole-resistant *Candida albicans* isolate in a vaginal model. *J Antimicrob Chemother* (2009); 64(3):571–3.

[12] **Sobel JD, Kapernick PS, Zervos M, et al.** Treatment of complicated *Candida* vaginitis: Comparison of single and sequential doses of fluconazole. *Am J Obstet Gynecol* (2001); 185(2):363–9.

[13] **Pfaller MA, Diekema DJ, Jones RN, et al.** International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY Antimicrobial Surv. *J Clin Microbiol* (2001); 39(9):3254–9.

[14] **Pfaller MA, Diekema DJ, Messer SA, et al.** Activities of fluconazole and voriconazole against 1,586 recent clinical isolates of *Candida* species determined by Broth microdilution, disk diffusion, and Etest methods: report from the ARTEMIS Global Antifungal Susceptibility Program, 2001. *J Clin Microbiol* (2003); 41(4):1440–6.

[15] **Kamath P, Pais M, Nayak MG**. Risk of vaginal candidiasis among pregnant women. *Int J Curr Microbiol Appl Sci* (2013); 2(9):141–6.

[16] **Kalo-Klein A, Witkin SS**. *Candida albicans*: Cellular immune system interactions during different stages of the menstrual cycle. *Am J Obstet Gynecol* (1989); 161(5):1132–6.

[17] **Špaček J, Buchta V, Jílek P, et al.** Clinical aspects and luteal phase assessment in patients with recurrent vulvovaginal candidiasis. *Eur J Obstet Gynecol Reprod Biol* (2007); 131(2):198–202.

[18] **Keller MJ, Guzman E, Hazrati E, et al.** PRO 2000 elicits a decline in genital tract immune mediators without compromising intrinsic antimicrobial activity. *AIDS* (2007); 21(4):467–76.

[19] **Madani ND, Malloy PJ, Rodriguez-Pombo P, et al.** *Candida albicans* estrogen-binding protein gene encodes an oxidoreductase that is inhibited by estradiol. *Proc Natl Acad Sci* (1994); 91(3):922–6.

[20] **Malloy PJ, Zhao X, Madani ND, et al.** Cloning and expression of the gene from *Candida albicans* that encodes a high-affinity corticosteroid-binding protein. *Proc Natl Acad Sci* (1993); 90(5):1902–6.

[21] **Nohmi T, Abe S, Dobashi K, et al.** Suppression of anti- *Candida* activity of murine neutrophils by progesterone in vitro : a possible mechanism in pregnant women's vulnerability to vaginal candidiasis. *Microbiol Immunol* (1995); 39(6):405–9.

[22] **Alves CT, Silva S, Pereira L, et al.** Effect of progesterone on *Candida albicans* vaginal pathogenicity. *Int J Med Microbiol* (2014); 304(8):1011–7.

[23] **Banerjee D, Martin N, Nandi S, et al.** A genome-wide steroid response study of the major human fungal pathogen *Candida albicans*. *Mycopathologia* (2007); 164(1):1–17.

[24] **Gujjar PR, Finucane M, Larsen B**. The effect of estradiol on *Candida albicans* growth. *Ann Clin Lab Sci* n.d.; 27(2):151–6.

[25] **Cheng G, Yeater KM, Hoyer LL**. Cellular and molecular biology of *Candida albicans* estrogen response. *Eukaryot Cell* (2006); 5(1):180–91.

[26] **Kurakado S, Kurogane R, Sugita T**. 17-β-Estradiol inhibits estrogen binding protein-mediated hypha formation in *Candida albicans*. *Microb Pathog* (2017); 109:151–5.

[27] **O'Connor C, Essmann M, Larsen B**. 17-β-estradiol upregulates the stress response in *Candida albicans*: implications for microbial virulence. *Infect Dis Obstet Gynecol* (1998); 6(4):176–81.

[28] **Williams DW, Wilson MJ, Lewis MAO, et al.** Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. *J Clin Microbiol* (1995); 33(9):2476–9.

[29] **Guyton A, Hall J**. Female physiology before pregnancy and female homones. *Textb. Med. Physiol.* 11th edn. Amsterdam: Elsevier; (2006). pp. 1011–26.

[30] **Silva S, Henriques M, Martins A, et al.** Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* (2009); 47(7):681–9.

[31] **Silva S, Henriques M, Oliveira R, et al.** *In vitro* biofilm activity of non-*Candida albicans Candida* species. *Curr Microbiol* (2010); 61(6):534–40.

[32] **CLSI**. *Reference method for broth dilution antifungal susceptibility testing of yeasts*. 4th ed. CLSI standard M27. Wayne, PA: Clinical and Laboratory Standards Institute; (2017).

[33] **Danby CS, Boikov D, Rautemaa-Richardson R, et al.** Effect of pH on in vitro susceptibility of *Candida glabrata* and *Candida albicans* to 11 antifungal agents and implications for clinical use. *Antimicrob Agents Chemother* (2012); 56(3):1403–6.

[34] **Schmidt P, Walker J, Selway L, et al.** Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*. *Proteomics* (2008); 8(3):534–44.

[35] **Amouri I, Sellami H, Borji N, et al.** Epidemiological survey of vulvovaginal candidosis in Sfax, Tunisia. *Mycoses* (2011); 54(5):e499-505.

[36] **Grigoriou O, Baka S, Makrakis E, et al.** Prevalence of clinical vaginal candidiasis in a university hospital and possible risk factors. *Eur J Obstet Gynecol Reprod Biol* (2006); 126(1):121–5.

[37] **Harriott MM, Lilly EA, Rodriguez TE, et al.** *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology* (2010); 156(Pt 12):3635–44.

[38] **Donlan RM, Costerton JW**. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* (2002); 15(2):167–93.

[39] **White S, Larsen B**. *Candida albicans* morphogenesis is influenced by estrogen. *Cell Mol Life Sci* (1997); 53(9):744–9.

[40] **Uppuluri P, Chaturvedi AK, Srinivasan A, et al.** Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog* (2010); 6(3):e1000828.

[41] **Chassot F, Negri MFN, Svidzinski AE, et al.** Can intrauterine contraceptive devices be a *Candida albicans* reservoir? *Contraception* (2008); 77(5):355–339.

[42] **Lee W, Lee DG**. A novel mechanism of fluconazole: fungicidal activity through dose-dependent apoptotic responses in *Candida albicans*. *Microbiology* (2018); 164(2):194–204.

[43] **Greer ND**. Voriconazole: the newest triazole antifungal agent. *Baylor Univ Med Cent Proc* (2003); 16(2):241–8.

[44] **CLSI**. *Performance standards for antifungal susceptibility testing of yeasts*. 1st ed. CLSI standard M60. Wayne, PA;

(2017).

[45] **Zhang X, Essmann M, Burt ET, et al.** Estrogen effects on *Candida albicans*: a potential virulence-regulating mechanism. *J Infect Dis* (2000); 181(4):1441–6.

[46] **Uppuluri P, Chaffin WL**. Defining *Candida albicans* stationary phase by cellular and DNA replication, gene expression and regulation. *Mol Microbiol* (2007); 64(6):1572–86.

[47] **Spitzer M, Wiederhold NP**. Reduced antifungal susceptibility of vulvovaginal *Candida* species at ormal vaginal pH levels. *J Low Genit Tract Dis* (2018); 22(2):152–8.

[48] **NCCLS**. Reference method for broth dilution antifungal susceptibility testing of yeasts ; Approved Standard — Second Edition. *NCCLS Doc M27-A2* (2002); 22(15).

[49] **Rubio MC, de Ocáriz IR, Gil J, et al.** Potential fungicidal effect of voriconazole against *Candida spp*. *Int J Antimicrob Agents* (2005); 25(3):264–7.

[50] **Hawser S**. Comparisons of the effects of fungicidal and fungistatic antifungal agents on the morphogenetic transformation of *Candida albicans*. *J Antimicrob Chemother* (1999); 43(3):411–3.

[51] **Anaissie EJ, Kontoyiannis DP, Huls C, et al.** Safety, plasma concentrations, and efficacy of high-dose fluconazole in invasive mold infections. *J Infect Dis* (1995); 172(2):599–602.

[52] **Larsen B, White S**. Antifungal effect of hydrogen peroxide on catalase-producing strains of *Candida spp*. *Infect Dis Obstet Gynecol* (1995); 3(2):73–8.

[53] **Cuéllar-Cruz M, Briones-Martin-del-Campo M, Cañas-Villamar I, et al.** High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot Cell* (2008); 7(5):814–25.

[54] **Seneviratne CJ, Wang Y, Jin L, et al.** *Candida albicans* biofilm formation is associated with increased anti-oxidative capacities. *Proteomics* (2008); 8(14):2936–47.

[55] **Ramage G, Bachmann S, Patterson TF, et al.** Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J Antimicrob Chemother* (2002); 49(6):973–80.

[56] **Seneviratne CJ, Wang Y, Jin L, et al.** Proteomics of drug resistance in *Candida glabrata* biofilms. *Proteomics* (2010); 10(7):1444–54.

Chapter 5

Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: a transcriptomic analysis

Main goal

To study the modulation of *Candida albicans* biofilm genomic profile and to analyse target gene regulation of transcription factors responsive to progesterone and acidic conditions.

Conclusions

This study showed that the regulation of biofilm gene expression by progesterone at acidic conditions is a complex process that involves relevant virulence regulators, which are potentially implied in *C. albicans* vaginal pathogenicity.

This chapter is based on the following article:

Gonçalves B, Bernardo R, Wang C, Pedro NA, Butler G, Azeredo J, Henriques M, Mira NP, Silva S. Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: a transcriptomic analysis. *International Journal of Medical Microbiology* (2019) (in review).

5.1 Introduction

Vulvovaginal candidiasis (VVC) affects millions of women every year and is considered to be an important public health problem [1]. Although VVC is not usually a life-threatening condition, the vaginal tract constitutes a main access route to the bloodstream. Infections of this niche therefore have the potential to result in a severe disseminated infection, particularly in immunocompromised patients [2]. Most (if not all) women carry *Candida* cells in the vaginal tract at some point in their lives, with or without symptoms of infection [3]. Despite an increased identification of non-*Candida albicans Candida* species (NCAC) [4,5], *Candida albicans* is still the most common species identified in women with VVC [6–8]. The ability of *C. albicans* to form biofilms is an important virulence factor as it confers unique characteristics compared to its planktonic counterpart cells, including significant resistance to antifungal agents, host defence mechanisms and physical and chemical stress [9]. In the vaginal environment, *Candida* species can form biofilms on the vaginal epithelium [10] and also on intrauterine devices (IUDs) thereby promoting VVC [11,12]. The ability of *Candida* species to form biofilms in the vaginal environment is an important clinical problem, since the recalcitrant nature of biofilms to currently used antifungals prevents definitive eradication of these microbes from the vaginal lumen thus contributing to the recurrence of VVC [10].

The development of VVC has been associated with the disturbance of the hormonal vaginal environment due to behavioural or host-related factors, including pregnancy [1]. It is thought that the high levels of progesterone secreted in pregnancy may contribute to VVC development by stimulating the production of glycogen by epithelial cells [13,14] and inhibiting the host immune response [15]. Furthermore, progesterone has direct effects on the physiology of *Candida* cells, potentially mediated by the stimulation of *Candida* receptors for progesterone [16]. A transcriptional survey of the effect of progesterone on *C. albicans* planktonic cells identified activation of stress response pathways, including the induction of genes involved in host immune and drugs response [17]. This study also reported that progesterone decreases the drug susceptibility of *C. albicans* planktonic cells [17]. Additionally, a previous study [18] showed that progesterone, at pregnancy levels, reduces the ability of *C. albicans* strains to form biofilms. This unexpected finding fostered the present work in which it is aimed to deep the current understanding on how progesterone modulates the process of *C. albicans* biofilm formation at the vaginal acidic pH, something that has not been examined before and that is essential for a full understanding of the pathogenesis of this species in the acidic vaginal tract.

5.2 Materials and Methods

5.2.1 Strains and initial culture conditions

The reference strain *C. albicans* SC5314 was the main strain used in this work. Additionally, some experiments were carried out using *C. albicans* mutant strains and their respective parental strain. All the strains are listed in Table 5.1.

For all the experiments the strains were grown in Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h, followed by 18 h in Sabouraud dextrose broth (SDB; Merck, Darmstadt, Germany), as described in Chapter 2 - section 2.2.1. For the subsequent planktonic growth or biofilm development, the cells were suspended in Roswell Park Memorial Institute medium (RPMI; Sigma-Aldrich, St Louis, MO, USA) medium at pH 4 and the cellular density was estimated using a Neubauer haemocytometer. The experiments described in next sections were performed in triplicate (same pre-inoculum) and in three independent assays (pre-inoculums independently prepared).

A stock solution of progesterone (Sigma-Aldrich, St Louis, MO, USA) at 10 mM was prepared on ultrapure water and stored at -20°C to be used in all experiments.

Table 5.1 *Candida albicans* strains used in this study

Strain name	Parent	Relevant genotype	Reference
SC5314		Prototrophic	Lab collection
SN76	SC5314	*arg4Δ/arg4Δ, his1Δ/his1Δ, ura3Δ-iro1Δ::λimm^{434}/ura3Δ-iro1Δ::λimm^{434}*	[19]
sfl1Δ/sfl1Δ	SN76	*sfl1Δ::ARG4/sfl1Δ::HIS1*	[20]
tec1Δ/tec1Δ	SN76	*tec1Δ::ARG4/tec1Δ::HIS1*	[20]
brg1Δ/brg1Δ	SN76	*brg1Δ::ARG4/brg1Δ::HIS1*	[20]

5.2.2 Planktonic growth analysis

For the planktonic growth, cellular suspensions of *Candida albicans* SC5314 were prepared at $1x10^5$ cells/ml in RPMI at pH 4, either supplemented or not with 2 μM of progesterone [18]. The

suspensions were placed in 25 mL Erlenmeyer flasks, maintained at 37°C with agitation (120 rev/min) and the increase in optical density at 690 nm was measured over time using a microtiter plate reader (Bio-Tek Synergy HT, Izasa, Winooski, VT, USA). The results were presented as optical density over 30 h of growth.

5.2.3 Biofilm formation and analysis

To study the effect of progesterone on biofilm formation, biofilms were developed in the presence and absence of progesterone as described by Alves et al. [18]. Briefly, suspensions of *C. albicans* SC5314 cells were prepared at $1x10^5$ cells/ml in RPMI at pH 4, either supplemented or not with 2 µM of progesterone, and placed into wells of 96-wells polystyrene microtiter plates (Orange Scientific, Braine-l`Alleud, Belgium) (200 µl per well). The plates were incubated for 24 h at 37°C under agitation (120 rev/min). After incubation, the medium was aspirated and non-adherent cells were removed by washing the biofilms with of PBS. The biofilms were analysed as described in the next sections.

5.2.3.1 Biomass and biofilm cells' cultivability analysis

The biofilms' total biomass was analysed by Crystal Violet (CV) staining methodology [21] and the number of cultivable cells into biofilms by CFU counting methodology [22], as described in Chapter 2 - sections 2.2.4. and 2.2.5, respectively. The results were presented per unit area of biofilm.

5.2.3.2 Metabolic activity

A XTT reduction assay [23] was used to determine the metabolic activity of *C. albicans* SC5314 biofilms formed in the presence and absence of progesterone. A 200 µl aliquot of a solution containing 100 µg/µl of XTT (2,3-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (Sigma-Aldrich, St Louis, MO, USA) and 10 µg/µl of PMS (phenazine methosulfate) (Sigma-Aldrich, St Louis, MO, USA) was added to wells with developed biofilms. The plates were incubated for 3 h in the dark, at 37°C under agitation (120 rev/min). Colorimetric changes were measured at 490 nm using a microtiter plate reader. The absorbance values were normalized with respect to the CFUs and are presented as Absorbance XTT/Log (CFUs/ml).

5.2.3.3 Biofilm structure

The structure of *C. albicans* SC5314 biofilms formed with and without progesterone was analysed by scanning electron microscopy (SEM) [18]. Biofilms were formed as described above, except on 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) (1 ml of cell suspension per well). Developed biofilms were dehydrated with ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min), air dried for 20 min and placed in a desiccator until analysis. Before observation, the base of the wells was removed and mounted onto aluminum stubs, sputter coated with gold. Biofilms were then imaged with an S-360 scanning electron microscope (Leo, MA, Cambridge, USA).

5.2.3.4 Biofilm matrix extraction and analysis

In order to study the effect of progesterone on biofilm matrix production, biofilms of *C. albicans* SC5314 were formed in the presence and absence of progesterone as described above, using 24-well polystyrene microtiter plates. The biofilms were washed, scrapped from the wells with PBS and separated from their matrices by sonication followed by centrifugation, as described in Chapter 2 - section 2.2.8.

The matrix protein content was measured using the BCA Kit (Bicinchoninic Acid, Sigma-Aldrich, St Louis, MO, USA) as described in Chapter 2 - section 2.2.8.1. Total carbohydrate content was estimated with the phenol-sulfuric method, according to a previous described procedure [24], as described in Chapter 2 - section 2.2.8.2. The results were normalized with the biofilm cells' dry weight, achieved by drying, at 37°C, the biofilm cells separated from the matrices, until a constant weight was obtained [25], and were presented as mg of protein or carbohydrate per g of biofilm dry weight ($mg/g_{biofilm}$).

5.2.3.5 Biofilm cells' susceptibility testing

The susceptibility of *C. albicans* biofilm cells to fluconazole (Pfizer, New York, NY, USA) was tested using the reference protocol for broth microdilution antifungal susceptibility testing of yeasts, according to the Clinical and Laboratory Standards Institute methods [26]. Biofilms developed as described above, in the presence and absence of progesterone, were scraped from the wells with PBS and the suspensions were vigorously vortexed for 2 min and centrifuged at 5000 g for 5 min at 4°C.

Pellets were resuspended in RPMI at pH 4 and the cell suspensions were used to the antifungal susceptibility tests and minimal inhibitory concentrations (MICs) determination, according to the reference method [26].

5.2.4 Transcriptomic analysis

The effect of progesterone on the transcriptome of *C. albicans* biofilms was assessed using species-specific DNA microarrays [27]. For this, the transcriptomes of *C. albicans* SC5314 cells present in biofilms grown for 24 h in the absence and presence of progesterone (2 μM) were compared with the transcriptome of planktonic cells cultivated for the same time in hormone-free RPMI medium (pH 4). The experimental setups used to cultivate the cells in planktonic and biofilm life styles were the same as described above and using 24-well polystyrene microtiter plates (1 ml per well). Developed biofilms obtained after 24 h of cultivation in the presence and absence of progesterone were washed and scraped from the plates with PBS and the suspensions were sonicated (Ultrasonic Processor, Cole-Parmer, IL, USA) for 30 s at 30 W, to separate the cells from the biofilm matrix [21]. Then, all cell suspensions (planktonic and biofilm-forming cells) were centrifuged at 3000 g for 10 min at 4°C, the supernatants were rejected and pellets were used for RNA extraction.

5.2.4.1 RNA extraction

Total RNA extraction was performed using the RiboPure – Yeast Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions, but with some alterations in order to maximize the quantity and quality of the obtained RNA. Briefly, *C. albicans* pellets were resuspended in lysis buffer, 10% SDS and a mixture of phenol:chloroform:IAA. The mixtures were vortexed vigorously for 15 s, transferred to screw cap tubes containing cold Zirconia beads and homogenized for 10 min with the vortex set at maximum speed. The obtained lysates were centrifuged for 5 min at 16100 g at room temperature, to separate the aqueous phase, containing the RNA, from the organic phase. Binding buffer and 100% ethanol were added to the aqueous phase and the mixture was applied to filter cartridges assembled in collection tubes, which were centrifuged for 1 min to pass the mixture through the filter. After that, the filters were washed with wash solutions (three times), with centrifugations of 1 min to pass each wash solution through the filter. Then, the filters were centrifuged for 1 min to remove the excess of wash solutions and were transferred to fresh collection tubes. Then, the RNA was eluted

in two times by applying elution solution (preheated to 95°C) to the filter and centrifuging for 1 min. To remove contaminating chromosomal DNA from the isolated RNA, a DNase digestion reaction was assembled at room temperature (RNA sample, DNase Buffer and DNase I) and incubated for 30 min at 37°C. Then, DNase Inactivation Reagent was added to the mixture and allowed to react for 5 min at room temperature. Lastly, the samples were centrifuged for 3 min at 21000 g to pellet the DNase inactivation reagent and the RNA (supernatant) was transferred to a fresh tube. RNA concentration and purity in each sample was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, CA, USA).

5.2.4.2 Microarrays analysis

cDNA synthesis, hybridization and scanning were performed using protocols similar to those described previously [28], with the exception of that hybridization was carried out using an Agilent hybridization oven at 65°C for 17 h at 100 rpm. In brief, 6 µg of total RNA was incubated with 1.4 µg of anchored Oligo(dT)$_{20}$ primer (Invitrogen, Carlsbad, CA, USA) in a total volume of 18.5 µl for 10 min at 70°C. First-strand buffer (Invitrogen, Carlsbad, CA, USA), 0.5 mMdATP, dTTP, and dGTP; 50 µM dCTP; 10 mM dithiothreitol; 2 µl Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA); and 2 µl Cy3-dCTP or Cy5-dCTP (Amersham, PA53021 and A55021) were added to a total volume of 40 µl, incubated at 42°C for 2 h followed by 1 h at 42°C with an additional 1 µl of Superscript III. RNA was degraded by addition of 1 µl of RNase A at 50 µg/mL and 1 µl of RNase H at 1 unit/µl and incubated at 37°C for 30 min. The labelled cDNAs were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), using a modified protocol. Samples were prepared for hybridization using Agilent's Two-Color Microarray-Based Gene expression (Quick Amp labelling protocol) and the Gene Expression Hybridization Kit. 10 µl each Cy3-labeled and Cy5-labeled cDNA were used per array (8 X 15 k, design ID 065138), in a total volume of 50 µl. Hybridized microarrays were scanned with an Axon 4000B scanner (Axon Instruments, Union City, CA, USA) and data were acquired with GenePix Pro 5.1 software (Axon Instruments, Union City, CA, USA). Data was analysed using the LIMMA package in Bioconductor (www.bioconductor.org). Lowess normalization and background correction were applied to each array separately, and quantile normalization was used to allow log ratios to be compared across arrays. Only genes exhibiting $\log_2$FC >1.0 and having an associated P-value below 0.01 were selected for further analysis.

5.2.5 Measurement of gene transcription based on quantitative real-time PCR (qRT-PCR)

In order to validate some of the results obtained in the microarray analysis, the expression profile of a set of specific genes (*TEC1*, *CRZ2* and *CDR1*) was obtained using qRT-PCR. The experimental conditions used to cultivate *C. albicans* SC5314 cells and to obtain RNA were the same as those described above for the transcriptomic analysis. Full-length gene sequences were obtained from the *Candida* Genome Database [29] and the respective primers were designed using Primer3 web software (the sequences are provided in Table AIII.1 – Annex III). The specificity of each primer was confirmed using BLAST [30] and also applying a PCR to genomic DNA extracted from *C. albicans* SC5314 planktonic cells, using the various primer pairs. The procedures to obtain the complementary DNA (cDNA) and to perform qRT-PCR were the same as described in Chapter 3 - section 3.2.2.6., using the transcript level of *ACT1* mRNA as an internal control. The Ct value of each sample was determined and the relative gene expression levels calculated using the ΔCt method [31], being normalized with the internal control gene ($Ct_{average}$ = 23.02 ± 0.35).

5.2.6 Experiments using *C. albicans* mutant strains

In order to investigate the role of some genes (*SFL1, TEC1* and *BRG1*) suggested by our microarray analysis to be important to the biofilm formation in the specific conditions used in this study (pH 4 and presence of progesterone), *C. albicans* biofilms of homozygous null mutants were developed. Mutant and respective parental strains (auxotrophs) used for these experiments are listed in Table 5.1. Biofilms were developed as previously described for *C. albicans* SC5314 using RPMI at pH 4 supplemented or not with progesterone. Biofilms grown for 24 h were washed with PBS and imaged with ChemiDoc Image System (Bio-Rad, Hercules, CA, USA). Additionally, gene expression of mutants' biofilm cells was analysed by qRT-PCR. For that, biofilm formation, RNA extraction and qRT-PCR procedures were performed as described above for quantitative RT-PCR analyses of *C. albicans* SC5314. Primers for the target genes were designed using Primer3 web software and their sequences are provided in Table AIII.1 – Annex III.

5.2.7 Statistical analysis

Results of biofilm biomass, cells cultivability, metabolic activity, matrix composition and gene expression (qRT-PCR) were statistically analysed using t tests implemented in GraphPad Prism 6 software. All tests were performed with a confidence level of 95 %.

5.3 Results and discussion

5.3.1 Effect of progesterone on planktonic growth and biofilm formation

To obtain a clearer picture of the effects exerted by progesterone on the physiology of *C. albicans* it was examined how the presence of this hormone affects growth of the yeast cells, in either planktonic or biofilm-forming conditions. The assays were performed in RPMI medium at pH 4 in order to mimic the acidic vaginal environment (in the range of 3.6-4.5) [32]. Additionally, 2 μM of progesterone was used, which is the highest concentration reported in the plasma of pregnant women in the third trimester [18], as the hormonal disturbance promoted by pregnancy is considered an important risk factor for VVC development [1]. In planktonic conditions, the concentration of progesterone used had no significant effect on growth of *C. albicans* SC5314 cells comparatively to the growth curve obtained in unsupplemented RPMI medium (Figure AII.1 – Annex II). However, the presence of progesterone reduced the ability of *C. albicans* SC5314 to form biofilms, resulting in a statistically significant decrease on cell cultivability (P-value ≤0.0001) and total biomass (P-value ≤0.01) (Figure 5.1-A and B), compared to biofilms formed in the absence of the hormone. Specifically, a decrease in the number of cultivable cells of approximately 2 orders of magnitude [Log (CFUs/ml)/cm^2] (Figure 5.1-A) and a decrease in biofilm biomass of approximately 20% were observed (Figure 5.1-B). These results are consistent with those reported by Alves et al. [18]. It is important to stress that the study of Alves et al. [18] was undertaken using two strains (ATCC 90028 and 558234, a reference strain from the American Type Culture Collection (ATCC) and a vaginal isolate, respectively), different from the one used in this study, thereby leading us to conclude that the inhibitory effect of progesterone on biofilm formation by *C. albicans* is independent of the genetic background of the strains used.

The effect of progesterone on C*. albicans* biofilms' cells was also evaluated, using XTT reduction assay. The results obtained (Figure 5.1-C) show a slight decrease in the metabolic activity of biofilm cells cultivated in the presence of progesterone, compared to the absence of the hormone; however, this difference is not statistically significant (P-value >0.05). The effect exerted by progesterone on the structure and morphological characteristics of the biofilms formed by *C. albicans* was observed by SEM. In the absence of progesterone *C. albicans* SC5314 cells formed a multilayer and compact biofilm that covered the entire surface, constituted by a dense network of hyphal forms (Figure 5.1-D). Differently, in the presence of 2 μM of progesterone, the biofilm consisted of a discontinuous multilayer with a lower number of cells. These observations are consistent with the reduction in the number of cultivable cells (Figure 5.1-A) and total biomass (Figure 5.1-B) of *C. albicans* biofilms.

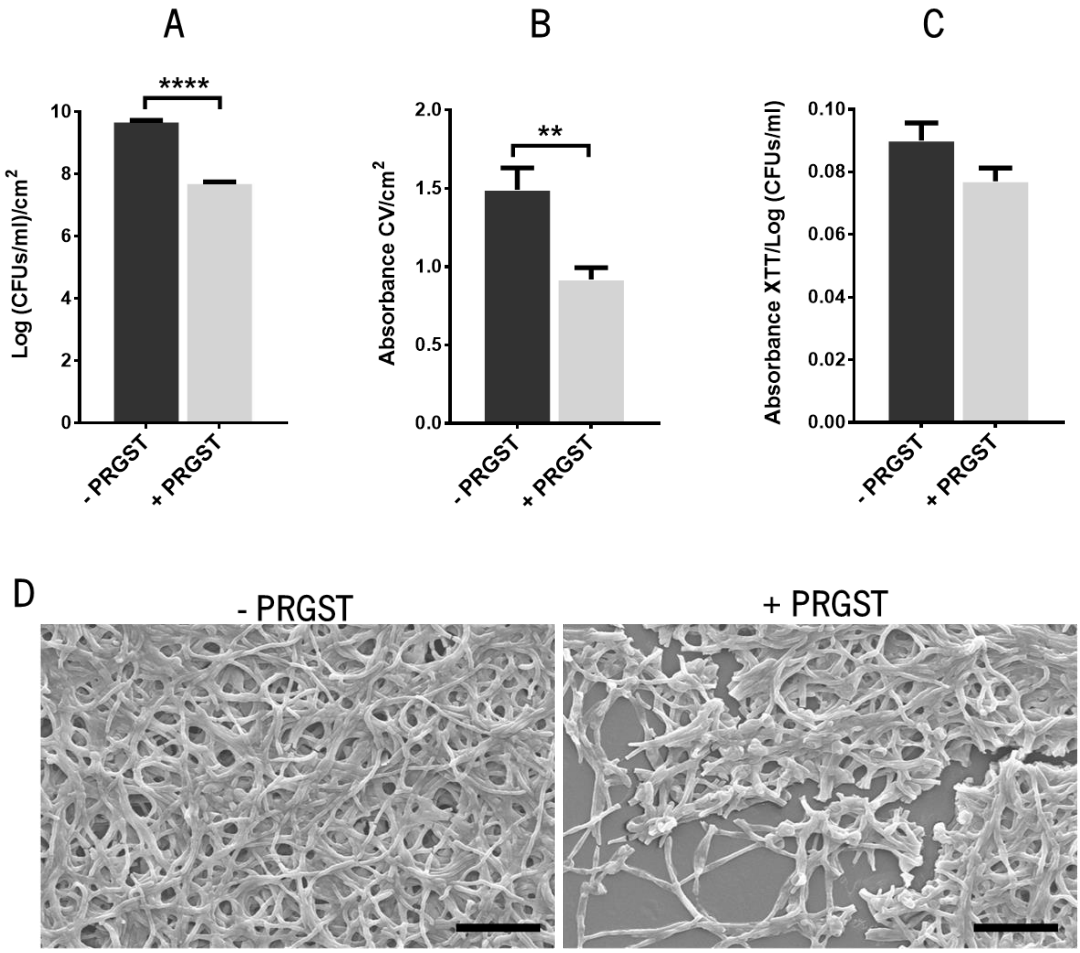


Figure 5.1 Effect of progesterone on *Candida albicans* biofilm formation. (A) Cell cultivability determination [Log (CFUs/ml)/cm^2], **(B)** total biomass quantification (Absorbance CV/cm^2), **(C)** metabolic activity measurement (Absorbance XTT/Log (CFUs/mL)) and **(D)** scanning electron microscopy images of *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4 in the absence (-PRGST) or presence of 2 μM of progesterone (+PRGST). Error bars represent standard deviation. Asterisks represent statistical difference between the conditions (**** P-value ≤0.0001; ** P-value ≤0.01). Original magnification of the images of panel D was x 1000 and the scale bars correspond to 20 μm.

5.3.2 Effect of progesterone on biofilm matrix composition

One of the most important characteristics of *Candida* biofilms is the presence and composition of the extracellular matrix. The functions of the matrix are not entirely clear, but it is thought that it controls the desegregation of the biofilm and protects against antifungal agents and the host immune system [33]. In order to analyse the effect of progesterone on *C. albicans* biofilm matrix composition, the total protein and carbohydrate contents of the extracellular matrix were determined in the absence and presence of progesterone. Table 5.2 shows that progesterone led to a decrease of total

carbohydrate to almost half of the amount detected in the matrix of the biofilm formed without progesterone (P-value ≤0.05). By contrast, there is an increase in the matrix protein level but it was not statistically significant (P-value >0.05) (Table 5.2). These results suggest that the biofilm matrix composition is changed in the presence of progesterone. In the absence of hormone, the quantities of protein and carbohydrate are close to that reported before for *C. albicans* biofilm matrix [34], although indicative of strain or pH influence. Previous studies with NCAC species have shown that biofilm matrix may be influenced by the species, strain and environmental conditions, being reported that an acidic pH may hamper the matrix production [21,35].

Table 5.2 Progesterone effect on *Candida albicans* biofilm matrix composition. Quantification of protein and carbohydrate (mg/g of biofilm dry weight) contents in the matrices of *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4, in the absence (- PRGST) or presence of 2 μM of progesterone (+PRGST). The results were normalized with the biofilm cells dry weight. Asterisks represent statistical difference when compared with biofilm formed in the absence of progesterone (*P-value ≤0.05).

	- PRGST	**+ PRGST**
Protein (mg/g $_{biofilm}$)	48.65 ± 4.19	69.77 ± 6.68
Carbohydrate (mg/g $_{biofilm}$)	162.60 ± 10.94	83.47 ± 11.17*

5.3.3 Transcriptional profiling of *C. albicans* cells present in biofilms formed in the presence or absence of progesterone

To gain further insights into the molecular mechanisms by which progesterone affects physiology and response of *C. albicans* SC5314 cells, were compared the transcriptomes of biofilms formed in the absence or presence of progesterone (after 24 h of cultivation in RPMI medium at pH 4, either supplemented or not with 2 μM of the hormone). Biofilm transcriptomes were also compared with the transcriptome of planktonic *C. albicans* SC5314 cells cultivated for 24 h in RPMI growth medium at pH 4. With this experimental setup it was possible to characterize the transcriptome of mature biofilms formed by *C. albicans* SC5314 in acidic conditions, something that, to the author's knowledge, had not been performed before, since previous studies were performed using biofilms formed in near-neutral pH [36–38]. Only genes whose transcripts increased by more than 2-fold in the

biofilm cells (in the presence or absence of progesterone), in comparison the expression levels registered in planktonic cells, were considered. A more detailed analysis on the observed transcriptome-wide alterations of *C. albicans* SC5314 biofilm cells follows.

5.3.3.1 Transcriptome-wide alterations in *C. albicans* biofilms in the absence of progesterone

Transcriptional profiling of *C. albicans* biofilms formed after 24 h of cultivation in acidic RPMI medium (at pH 4) showed a significant (P-value below 0.01) alteration (above or below 2-fold) in the expression of 1013 genes, comparing to planktonic cells. Specifically, 616 genes were up-regulated in biofilm cells, while 397 genes were down-regulated. A subset of the most relevant genes is listed in Table 5.3 and the full list is available in Table AIII.2 – Annex III. About 322 of the up-regulated genes were previously shown to be induced during biofilm formation by *C. albicans* [36–40], however, 294 genes are reported to be up-regulated in biofilm cells for the first time, according to the information available on *Candida* Genome Database (www.candidagenome.org) [29] (highlighted in grey in Table 5.3 and Table AIII.2 – Annex III). The identification of these new biofilm-induced genes in our dataset may reflect the acidic pH that was used in this work but not in previous transcriptomic profiling experiments of *C. albicans* biofilms [36–40]. Notably, 8 of the genes found be induced only in our study are essential for normal biofilm formation (highlighted in bold in Table 5.3), including *NDT80* and *WOR1*, encoding two transcriptional regulators [41,42]; *ALS5,* encoding an adhesin [43]; the membrane protein *PGA10*; *CSA2*, encoding a cell surface protein involved in iron utilization [44], and the poorly characterized genes *NPL3*, *C1_00160C_A* and *C7_00490C_A* [45]. The function of the additional genes with increased expression in the acidic biofilms (286) will require future study.

Table 5.3 Subset of genes found to be biofilm-regulated (up- and down-) in *Candida albicans* cells cultivated 24 h at pH 4. A subset of relevant genes whose expression was found to increase or decrease (above or below 2-fold) in *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4, in comparison with the transcript levels registered in planktonic cells cultivated in the same conditions, was selected and are herein shown, while the full list is available on Table AIII.2 – Annex III. Genes whose transcription was found to be biofilm-induced or –repressed specifically at the acidic conditions used in this study, are highlighted in grey and among them are highlighted in bold those previously described as essential to biofilm formation. The biological function indicated is based on the information available at *Candida* Genome Database.

Gene	Biological function	$\frac{\text{mRNA}_{\text{biofilm}}}{\text{mRNA}_{\text{planktonic}}}$
	Up-regulated genes	
ALS1	Cell-surface adhesin	20.17
ECE1	Candidalysin	18.12
CAN2	Basic aminoacid permease	16.91
SOD5	Cu-containing superoxide dismutase	15.01
C1_13130C_A	Putative histidine permease	12.59
COX3B	Cytochrome c oxidase involved in mitochondrial respiration	10.97
HGT2	Putative MFS glucose transporter	10.57
HYR1	Hyphal cell wall protein involved in biological adhesion	9.87
NAD1	NADH dehydrogenase involved in mitochondrial respiration	9.69
NAD3	NADH dehydrogenase involved in mitochondrial respiration	9.49
C2_10160W_A	Secreted protein	9.30
HGT1	High-affinity MFS glucose transporter	9.18
DIP5	Dicarboxylic amino acid permease	8.97
ATP6	Subunit of mitochondrial ATP synthase	7.84
PRA1	Cell surface protein that sequesters zinc from host tissue	5.88
QDR1	Putative antibiotic resistance transporter	5.71
C7_00490C_A	Putative AdoMet-dependent proline methyltransferase	2.93
NPL3	Putative RNA-binding protein	2.63
CSA2	Extracellular-associated protein involved in iron assimilation	2.58
C1_00160C_A	Putative nucleolar protein	2.57
ALS5	ALS family adhesin	2.38
NDT80	Meiosis-specific transcription factor	2.28
WOR1	Transcription factor of white-opaque phenotypic switching	2.16
PGA10	GPI anchored membrane protein	2.05
	Down-regulated genes	
DUR3	High affinity spermidine transporter	0.05
MEP2	Ammonium permease	0.09
JEN2	Dicarboxylic acid transporter	0.11
C2_00180C_A	Predicted uricase	0.15
C1_01630W_A	Putative mitochondrial complex I intermediate-associated protein	0.15
FDH1	Formate dehydrogenase	0.17
C1_07160C_A	Protein conserved among the CTG-clade	0.17
CWH8	Putative dolichyl pyrophosphate phosphatase	0.18
HSP30	Putative heat shock protein	0.18
LAP3	Putative aminopeptidase	0.18

Table 5.3 Continuation

Gene	Biological function	$\frac{\text{mRNA}_{\text{biofilm}}}{\text{mRNA}_{\text{planktonic}}}$
	Down-regulated genes	
CTN1	Carnitine acetyl transferase	0.18
CCP1	Putative Cytochrome-c peroxidase	0.18
MDH1-1	Predicted malate dehydrogenase precursor	0.18
C5_03770C_A	Putative formate dehydrogenase	0.18
DUR1,2	Urea amidolyase involved in response to abiotic stimulus	0.19
ADH2	Alcohol dehydrogenase;	0.19
PGA13	GPI-anchored cell wkhall protein involved in cell wall synthesis	0.19

The genes up and down-regulated in the acidic biofilms were functionally clustered using MIPS Functional catalogue (Figure 5.2). The functional classes enriched (P-value ≤0.001) in the dataset of up-regulated genes were "transcription", "protein synthesis", "protein with binding function" and "transport". On the other hand, down-regulated genes were enriched in genes involved in "metabolism" (including enrichment of the subclasses "amino acid", "nitrogen", "carbohydrate", "lipid/fatty acid" and "secondary" metabolisms), "generation of energy", "protein with binding function", "transport", "stress response" and "interaction with the environment" (Figure 5.2). In general, the functional clustering of the genes found to be differently expressed in our acidic biofilms is similar to those found in other studies [36–40], something that could be attributable to the fact that most of the genes that were found differentially expressed in our acidic biofilms have a poorly or even uncharacterized function.

To further understand the transcriptional regulatory network active in the formation of biofilms under acidic conditions, the PathoYeastract database [46] was used to cluster the up-regulated genes with transcription factors reported to control the process of biofilm formation in *C. albicans* (Ndt80, Efg1, Bcr1 and Tec1) [41]. Approximately 33% of the genes up-regulated in the acidic biofilms are documented targets of Ndt80 transcription factor, 32% of Efg1, 16% of Bcr1, 13% of Tec1 and 13% of Brg1 (Table AIII.3 – Annex III). Figure 5.3 shows the target genes of Efg1, Brg1, Bcr1 and Tec1 with differential expression under biofilm-forming conditions, according with the information available on the PathoYeastract database [46]. A significant overlap between the genes regulated by each transcription factor was observed, confirming the complex and intertwined nature of the regulatory network controlling expression of biofilm genes in *C. albicans* [41].

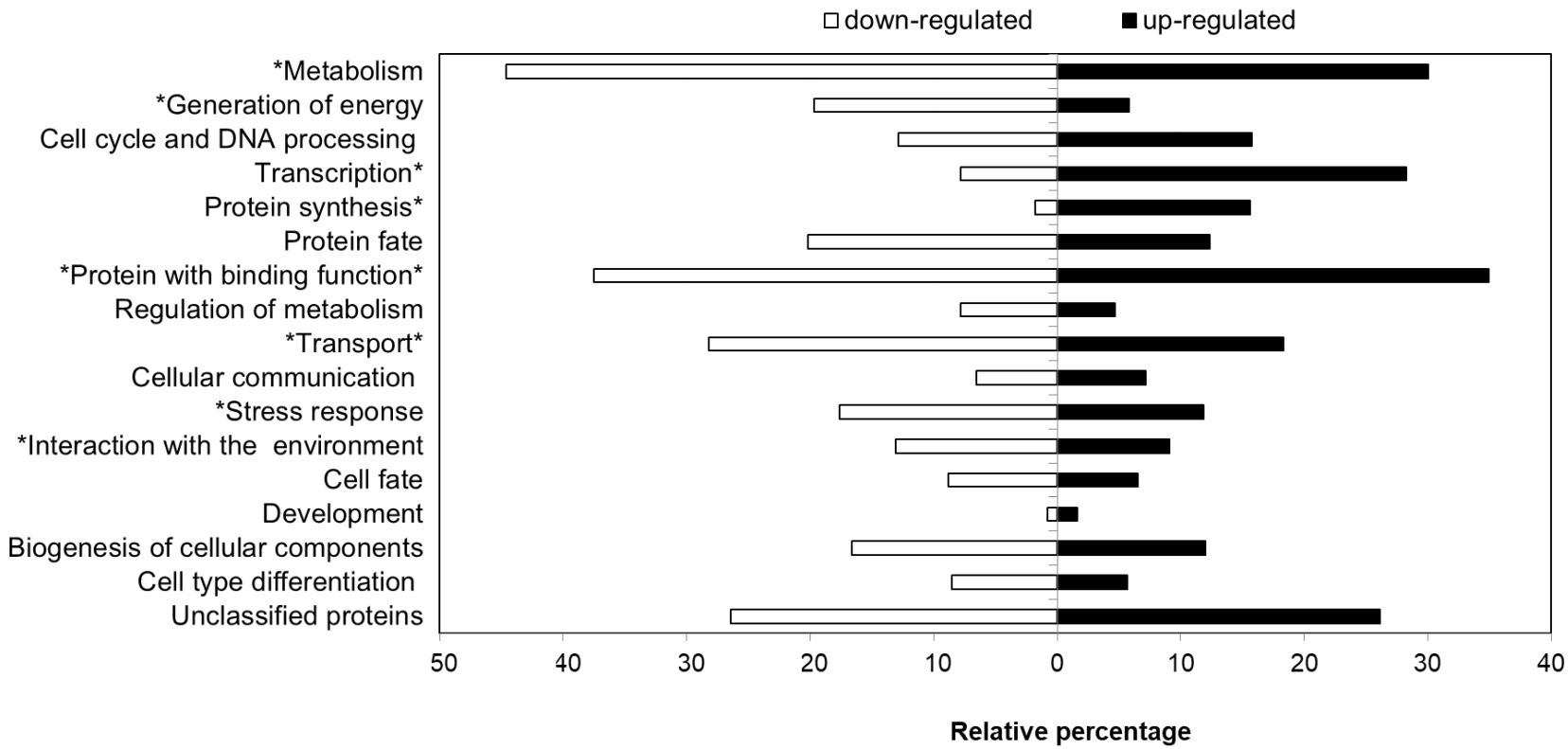


Figure 5.2 Functional distribution of genes found to be biofilm-regulated in *Candida albicans* cells cultivated for 24 h in RPMI at pH 4. The genes found to be up- or down-regulated in *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4, in comparison with the levels found in planktonic cells cultivated in the same conditions, were clustered according to their biological function, using MIPS Functional Catalogue database (black and white bars correspond to up- and down-regulated genes, respectively). The percentages shown correspond to the number of genes included in each functional class compared to the total number of up- or down-regulated genes. Functional categories considered significantly enriched in the datasets (P-value ≤0.001; taking into consideration the entire genome of *C. albicans* SC5314 reference strain) are indicated with asterisk (asterisk before or after the class denomination means enrichment among down- or up-regulated genes, respectively.

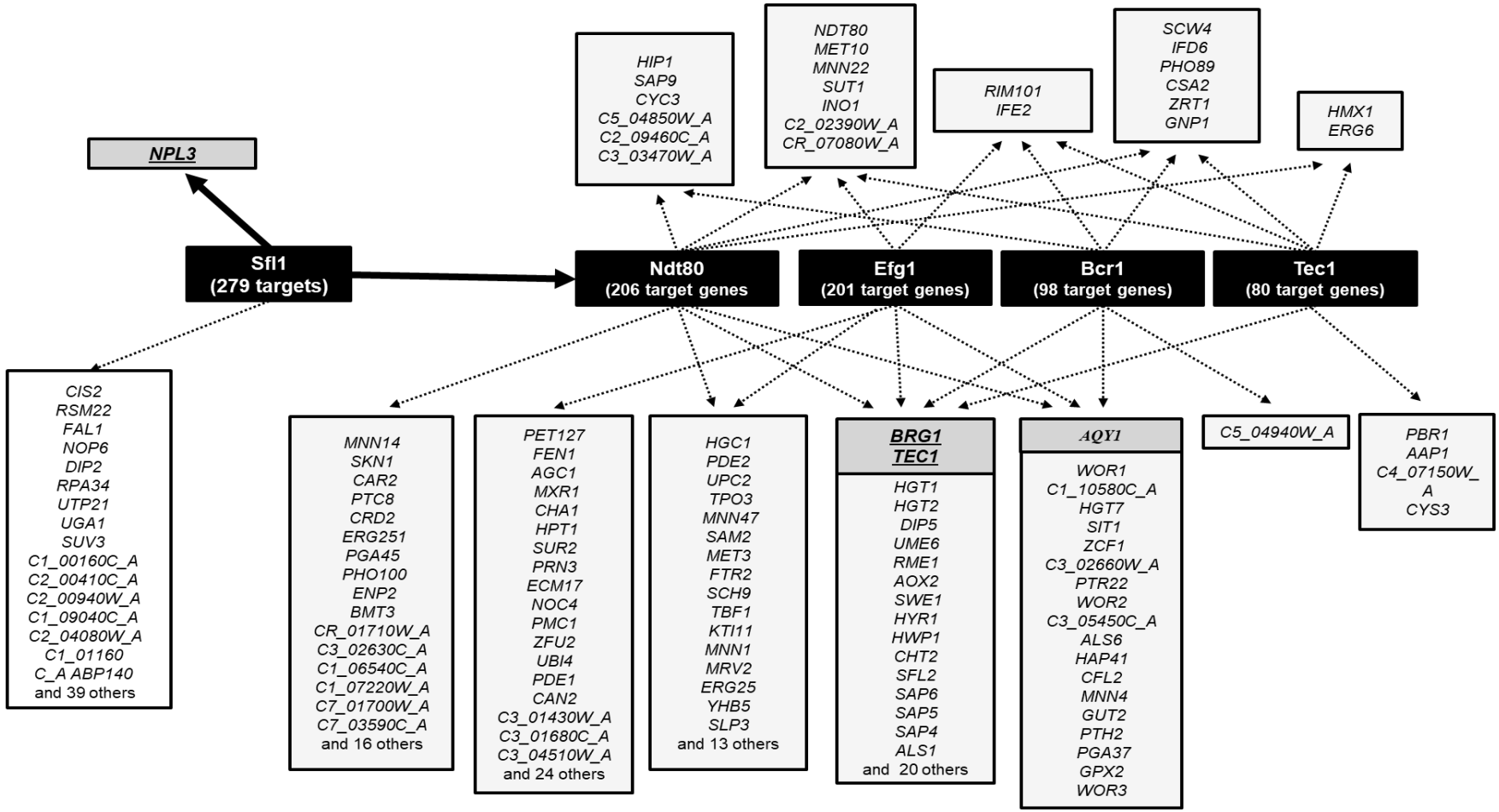


Figure 5.3 Proposed transcriptional regulatory network underlying the control of *Candida albicans* biofilm cells after 24 h of cultivation in RPMI at pH 4. The genes found to be up-regulated in *C. albicans* SC5314 biofilms formed after 24 h of cultivation in RPMI at pH 4, in comparison with the levels attained in planktonic cells, were clustered according with the existence of documented regulatory associations with transcription factors mediating the control of transcriptome-wide alterations related with biofilm formation, according with the information available on the PathoYeastract database. The number of documented targets attributed to each transcription factor is indicated in brackets inside the black boxes. Only genes having a biological function related with adhesion and/or biofilm formation (or described to be up-regulated under these conditions) were selected for this analysis, being identified in grey boxes those genes whose deletion was described to abolish biofilm formation. The results that gave rise to this figure are fully detailed in Table AIII.3 – Annex III.

The high percentage of documented targets of CaNdt80 (Figure 5.3) suggests that this regulator plays a particularly critical role in the control of expression under acidic conditions. However, 248 upregulated genes are not known targets of Efg1, Ndt80, Bcr1 and Tec1. Analysis using PathoYeastract suggests that 160 of these genes are targets of Sfl1. In fact, Sfl1 is predicted to regulate 279 genes in the entire dataset, which surpasses the number of targets attributed to Efg1, Ndt80, Bcr1 or Tec1 (Figure 5.3). Deletion of *SFL1* gene almost fully abolished biofilm formation prompted by *C. albicans* cells under acidic conditions (pH 4) (Figure 5.4-A), consistent with this regulator being a critical player in the reprogramming of genomic expression under those conditions. Recently, Sfl1 was found to be required for formation of microcolonies contributing to maximal adhesion to epithelial cells [47]. The involvement of Sfl1 in formation of acidic biofilms represents a further insight into the biological function of this regulator.

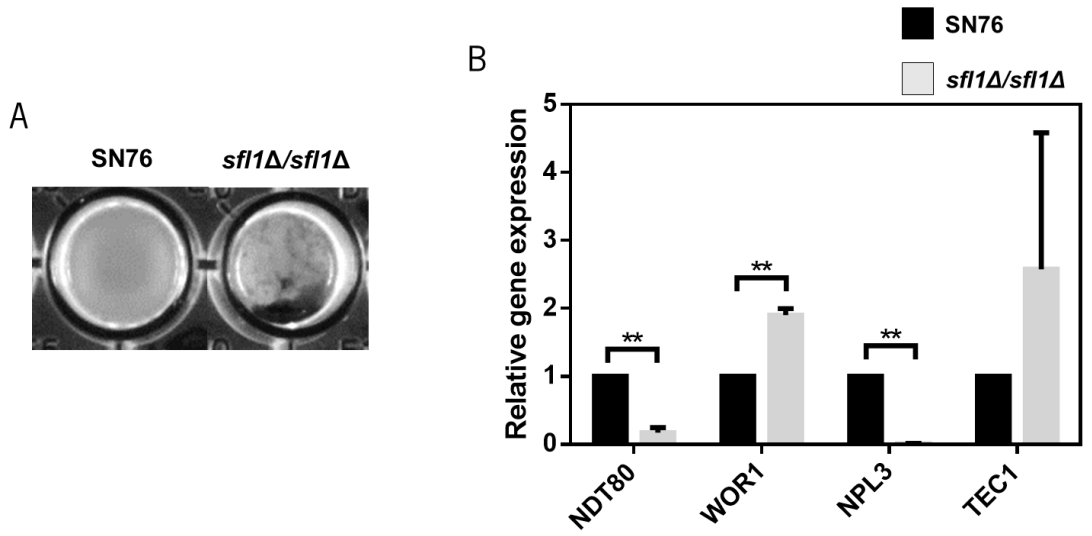


Figure 5.4 (A) Sfl1 is required for maximal *Candida albicans* biofilm formation under acidic conditions (pH 4); *C. albicans* homozygous mutant strain lacking *SFL1* (*sfl1Δ/sfl1Δ*) and the respective parental strain (SN76), were cultivated in RPMI at pH 4 for 24 h. The biofilms formed under these conditions were photographed and are shown. **(B)** Effect of Sfl1 expression in transcription of *NDT80*, *WOR1*, *NPL3* and *TEC1* in biofilm cells after 24 h of cultivation in RPMI under the same conditions as those used in the phenotypic assays shown in panel A. Error bars represent standard deviation. Asterisks represent statistical difference between the results of the mutant and parent strain. (** P-value ≤0.01).

The high overlap observed between the activated genes in our acidic biofilms documented to be regulated by Sfl1 and Ndt80 prompted us to test whether Sfl1 is a positive regulator of *NDT80* expression. In acidic biofilms formed by the mutant *sfl1Δ/sfl1Δ,* the expression of *NDT80* was found to be approximately 10% of the levels in the parental strain (Figure 5.4-B). Previous studies have also shown that there is functional interaction between Sfl1 and Ndt80 during hyphae formation in *C. albicans* [20]. Sfl1 was also found to be required for maximal expression of *NPL3* (Figure 5.4-B), which is up-regulated in acidic biofilms, and has been shown to be an important determinant of biofilm formation in *C. albicans* [45]. Further studies are required to fully characterize the role of Sfl1 in regulating gene expression during formation of acidic biofilms and, in particular, to identify Sfl1-regulated targets that are essential for biofilm formation. Although Sfl1 has been mostly described as a transcriptional repressor in *C. albicans* [47,48], evidences from chromatin immunoprecipitation profiling have shown that it also acts as a positive regulator [20]. Similarly, the ScSfl1 orthologue has also been found to have a dual effect acting both as a positive and a negative regulator [49]. The molecular mechanisms driving the regulation of the activity of Sfl1 under the different environmental contexts that may control its activity as an activator and/or repressor and how those environmental cues are transduced into that regulatory mechanism remain to be established.

5.3.3.2 Transcriptome-wide alterations in *C. albicans* biofilms in the presence of progesterone

Transcriptome-wide profiling of *C. albicans* biofilms obtained after 24 h of cultivation in RPMI medium (at pH 4) supplemented with 2 μM of progesterone identified a significant (P-value ≤0.01) alteration (above or below 2-fold) in the expression of 1057 genes (556 up- and 501 down-regulated), compared to expression in planktonic cells cultivated in the absence of the hormone (Table AIII.4 – Annex III). There is a large overlap between this dataset and the dataset of genes differentially expressed in the biofilms formed without progesterone (described above) (Figure 5.5). This indicates that most of the changes observed in the progesterone-exposed biofilms result from the process of biofilm formation itself and not from the direct exposure to the hormone.

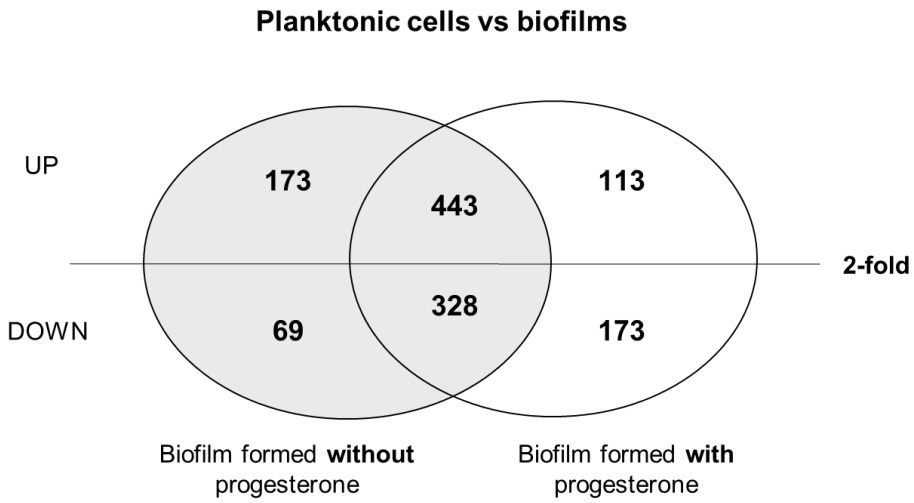


Figure 5.5 Venn diagram showing the number of *Candida albicans* genes whose transcription was found to be altered (up- or down-regulated) in biofilms formed with and/or without progesterone. Genes whose expression was found to increase or decrease (above or below 2-fold) in *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4, with and/or without 2 μM of progesterone, in comparison with the transcript levels registered in planktonic cells grown in hormone-free medium were selected for this analysis.

The genes showing at least 40% variation in the biofilms formed in the presence of the hormone, compared to the levels obtained in its absence, were characterized as progesterone-responsive. Using this criterion, 220 progesterone-responsive genes were identified which were then divided in four groups: *i)* genes up-regulated in biofilms formed in the presence or absence of progesterone, but showing a stronger induction in the presence of the hormone (42 genes); *ii)* genes more strongly up-regulated in biofilms without progesterone than in its presence (87 genes); *iii)* genes more strongly down-regulated in biofilms formed without progesterone (12 genes); *iv)* genes more strongly down-regulated in the progesterone-exposed biofilms (79 genes). A subset of relevant progesterone-responsive genes belonging to groups *i) a*nd ii) is shown in Table 5.4 and the full list is available in Table AIII.5 – Annex III.

Table 5.4 Subset of progesterone-responsive genes in *Candida albicans* SC5314 biofilms grown 24 h at pH 4. Was selected and listed here a subset of *C. albicans* SC5314 genes found be up-regulated in biofilms grown 24 h in RPMI at pH 4, in the presence (+ PRG) and/or absence (- PRG) of 2 μM of progesterone and whose expression differed more than 40% between the two biofilm-forming conditions. This table includes a subset of relevant genes found to be up-regulated in both conditions, but showing a stronger induction in the presence of the hormone (group *i*) and a subset of genes strongly up-regulated in biofilms without progesterone than on its presence (group *ii*). The full list of progesterone-responsive genes is available on Table AIII.5 – Annex III. The biological function indicated is based on the information available at *Candida* Genome Database.

Gene	Biological function	mRNA + PRG	mRNA - PRG	mRNA + PRG / mRNA - PRG
	Group *i)*			
ERG6	Protein involved in ergosterol biosynthesis	12.65	5.22	2.43
CDR2	Multidrug transporter of ABC superfamily	3.15	1.35	2.33
PGA31	Cell wall protein involved in response to drug	10.18	4.85	2.10
PGA45	Putative GPI-anchored cell wall protein	20.17	9.66	2.09
CDR1	Multidrug transporter of ABC superfamily	3.15	1.53	2.06
POT1	Putative peroxisomal 3-oxoacyl CoA thiolase	2.61	1.30	2.00
C3_02870C_A	Unknown function	4.74	2.49	1.90
GAP2	General amino acid permease	2.14	1.18	1.82
ATO1	Putative transmembrane protein	7.62	4.31	1.77
C7_02260W_A	Unknown function	3.23	1.85	1.74
INO1	Inositol-1-phosphate synthase	47.93	28.05	1.71
FAS2	Alpha subunit of fatty-acid synthase	2.43	1.44	1.68
C2_05980C_A	Putative acyl-CoA hydrolase activity	3.17	1.89	1.68
PTH2	Putative cAMP-independent regulatory protein	3.60	2.19	1.64
AGP3	Putative serine transporter	5.12	3.20	1.60
FAT1	Predicted enzyme of sphingolipid biosynthesis	2.12	1.33	1.59
GIT2	Putative glycerophosphoinositol permease	5.04	3.19	1.58
CSA2	Protein involved in iron assimilation	4.07	2.58	1.58
	Group *ii)*			
COX3B	Cytochrome c oxidase	1.54	10.97	0.14
NAD3	NADH dehydrogenase	1.38	9.49	0.15
ATP9	ATP synthase required for ATP synthesis	1.15	6.63	0.17
ATP6	ATP synthase required for ATP synthesis	1.52	7.84	0.19
NAD6	NADH dehydrogenase	1.69	8.54	0.20
COX2	Cytochrome c oxidase	1.76	8.56	0.21
WOR3	Transcription factor	0.85	2.63	0.32
SUT1	Transcription factor	0.96	2.16	0.44
BRG1	Transcription factor	0.89	2.01	0.44
RME1	Zinc finger protein; putative meiosis control	2.20	4.55	0.48
PRN3	Protein similar to pirin; unknown function	1.53	2.84	0.54
PRN1	Protein similar to pirin; unknown function	1.61	2.90	0.56
TEC1	Transcription factor	3.20	5.64	0.57
PGA11	Putative GPI-anchored protein	1.16	2.03	0.57
TPK1	cAMP-dependent protein kinase	2.32	3.86	0.60
GIT1	Glycero phosphodiester transporter	1.30	2.14	0.60

Table 5.4 Continuation

Gene	Biological function	mRNA + PRG	mRNA - PRG	mRNA + PRG / mRNA - PRG
	Group *ii)*			
WOR1	Transcription factor of phenotypic switching	1.32	2.16	0.61
IFE2	Putative alcohol dehydrogenase	1.50	2.41	0.62
SFL2	Transcription factor required for filament	1.79	2.84	0.63
HAP2	Transcription factor of low-iron induction	1.37	2.16	0.63
PBR1	Protein required for biofilm formation	2.51	3.92	0.64
SWE1	Putative protein kinase required for virulence	1.36	2.11	0.64
AHR1	Transcription factor involved in adhesion	1.76	2.62	0.67
HYR1	Hyphal cell wall protein involved in adhesion	6.82	9.87	0.69

Closer analysis of the set of genes with higher induction in the progesterone-exposed biofilms (clustered in group *i)* revealed a prominent increase in transcription of *CDR1* and *CDR2*, encoding two multidrug resistance transporters of the ABC Superfamily (Table 5.4). The up-regulation of *CDR1* in response to progesterone in *C. albicans* biofilm cells was further confirmed by qRT-PCR (Figure AII.2 – Annex II). Other studies have also reported up-regulation of *CDR1* transcription upon exposure of planktonic *C. albicans* cells to progesterone and to female serum [50,51]. Consistent with this observation, planktonic progesterone-exposed cells are less sensitive to fluconazole than cells cultivated in the absence of the hormone [17,51]. Similarly, in this study, cells extracted from progesterone-exposed biofilms were observed to be significantly more tolerant to fluconazole than cells from biofilms developed in the absence of the hormone (MIC of 1.5 μg/mL compared with 0.25 μg/mL). Additionally, many progesterone-responsive genes are involved in fatty acids metabolism and cellular respiration (Figure 5.6), what suggest some similarities with drug stress response [52], as also reported for *C. albicans* planktonic cells response to progesterone [17].

The inhibitory effect of progesterone in biofilm formation may be related to reduced expression of genes required for this process. Expression of 166 progesterone-responsive genes is reduced in biofilm cells grown with progesterone (87 clustered in group *ii* and 79 in group *iv*) (Table AIII.5 – Annex III). These include several key regulators of biofilm formation, including the transcription factors Tec1, Brg1, Ahr1, Wor1, Csr1 and Crz2 [41,42,53–55], the heat shock protein Hsp104 [56] and the uncharacterized protein Pbr1 [57] (Figure 5.6), among other genes previously shown to be required for biofilm formation (all highlighted in bold in Table AIII.5 – Annex III). The progesterone-decreased expression of *TEC1* and *CRZ2* genes in *C. albicans* biofilm cells was further confirmed by qRT-PCR (Figure AII.2 – Annex II).

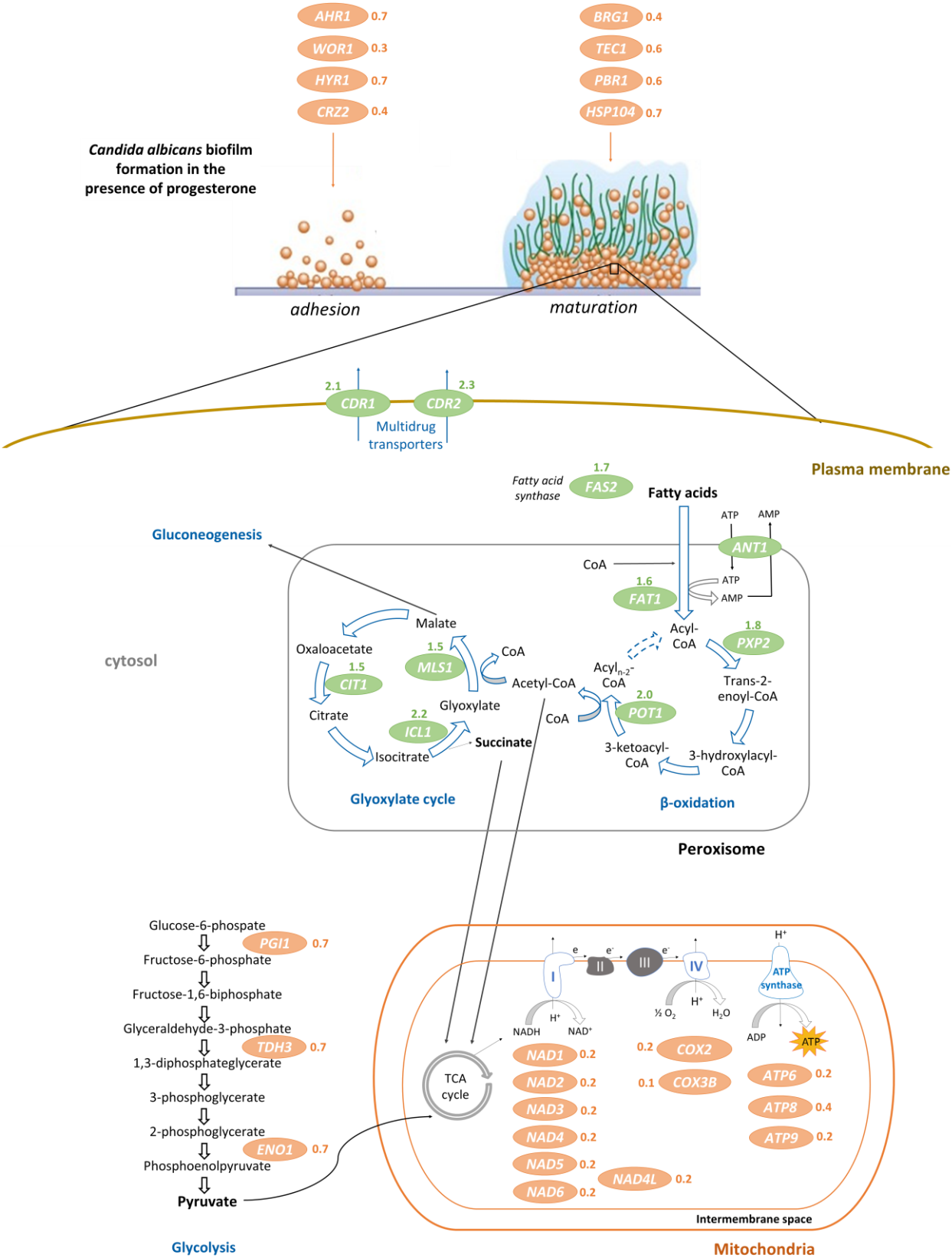
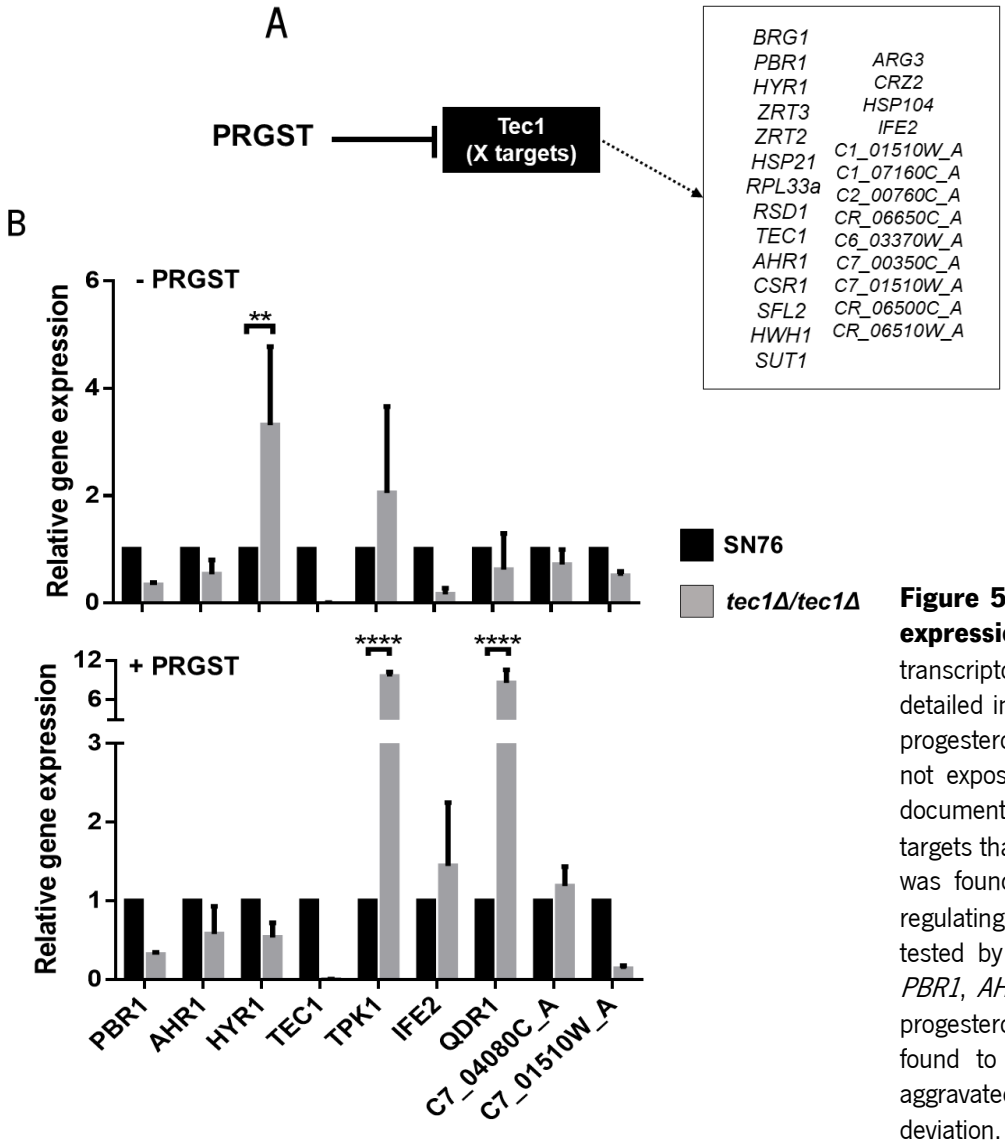


Figure 5.6 Schematic representation of the proposed genetic regulation of *Candida albicans* biofilm cells by progesterone. The scheme includes progesterone-responsive genes involved in adhesion, biofilm maturation, drug transport, fatty acids metabolism, glycolysis and mitochondrial respiration. The expression ratio found for each gene between biofilms formed with and without progesterone are given to it.

Several genes involved in the process of biofilm formation that had a reduced expression in the presence of progesterone are documented targets of Tec1 (Figure 5.7-A) and Brg1, according to the PathoYeastract database [46]. Therefore, it was tested whether these two regulators are required for biofilm formation in the presence of the hormone. Under the conditions that were used only the deletion of *TEC1* had a significant effect in formation of acidic biofilms, this phenotype being further aggravated in the presence of progesterone (Figure 5.7-C). Consistently, Tec1 was required for maximal expression of *PBR1* and *C7_01510W_A*, either in the presence or absence of progesterone (Figure 5.7-B). Tec1 is also required for maximal expression of *HYR1* in progesterone-exposed biofilm cells. However, in the absence of the hormone the expression of *HYR1* is increased in the *tec1Δ/tec1Δ* mutant strain. By contrast, the expression of *QDR1* presented a significant increase in the *tec1Δ/tec1Δ* mutant, only in progesterone-exposed biofilm cells (Figure 5.7-B). These observations are a clear reflection of the complex nature of the regulatory networks governing biofilm formation in *C. albicans*, which can be greatly shaped by the environmental conditions.

This study examined the alterations occurring in the genomic expression of *C. albicans* during biofilm formation at acidic conditions (pH 4) and in presence of progesterone, for the first time. Considering that the vaginal tract is one of the main driveways for the development of *C. albicans* infections, the identification of genes that may determine the ability of this yeast to survive in specific vaginal conditions may contribute to the disclosure of new targets to treat VVC infections.



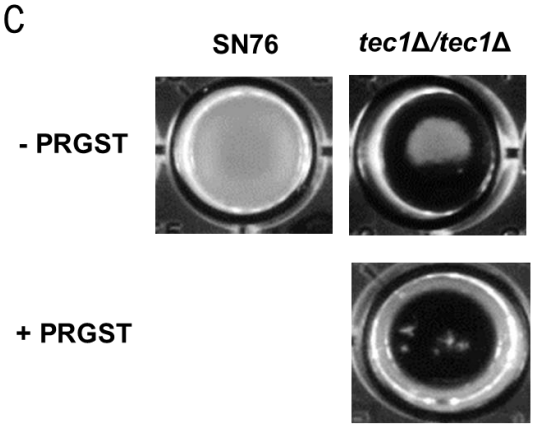


Figure 5.7 Tec1-documented targets that were found to have a reduced expression in progesterone-exposed biofilm cells (A and B). The transcriptome-wide analysis carried out led to the identification of a set of genes (fully detailed in Table AIII.5 – Annex III) that were found to have a reduced expression in progesterone-exposed biofilm cells, in comparison with the level attained in biofilms not exposed to the hormone. This set of genes included Tec1 and also a set of documented targets. **(A)** In the picture are depicted all those documented Tec1 targets that have a biological function related to biofilm formation or whose expression was found to be induced along biofilm formation. **(B)** The effect of Tec1 in up-regulating genes previously shown as relevant for *C. albicans* biofilm formation was tested by real-time PCR, being confirmed the positive effect in the expression of *PBR1*, *AHR1* and *C7_01510w_A*, both in absence of the hormone (-PRGST) and in progesterone-exposed biofilm cells (+PRGST). Consistent with these results, Tec1 was found to be essential for formation of acidic biofilms and this phenotype was aggravated in the presence of progesterone **(C)**. Error bars represent standard deviation. Asterisks represent statistical difference between the results of the mutant and parent strain. (**** P-value ≤0.0001; ** P-value ≤0.01).

References

[1] **Sobel JD**. Vulvovaginal candidosis. *Lancet* (2007); 369(9577):1961–71.

[2] **Ascioglu S, Rex JH, de Pauw B, et al.** Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. *Clin Infect Dis* (2002); 34(1):7–14.

[3] **Beigi RH, Meyn LA, Moore DM, et al.** Vaginal yeast colonization in nonpregnant women: a longitudinal study. *Obstet Gynecol* (2004); 104(5 Pt 1):926–30.

[4] **Ahmad A, Khan AU**. Prevalence of *Candida* species and potential risk factors for vulvovaginal candidiasis in Aligarh, India. *Eur J Obstet Gynecol Reprod Biol* (2009); 144(1):68–71.

[5] **Cetin M, Ocak S, Gungoren A, et al.** Distribution of *Candida* species in women with vulvovaginal symptoms and their association with different ages and contraceptive methods. *Scand J Infect Dis* (2007); 39(6–7):584–8.

[6] **Amouri I, Sellami H, Borji N, et al.** Epidemiological survey of vulvovaginal candidosis in Sfax, Tunisia. *Mycoses* (2011); 54(5):e499-505.

[7] **Fan SR, Liu XP, Li JW**. Clinical characteristics of vulvovaginal candidiasis and antifungal susceptibilities of *Candida* species isolates among patients in southern China from 2003 to 2006. *J Obstet Gynaecol Res* (2008); 34(4):561–6.

[8] **Vermitsky J-P, Self MJ, Chadwick SG, et al.** Survey of vaginal-flora *Candida* species isolates from women of different age groups by use of species-specific PCR detection. *J Clin Microbiol* (2008); 46(4):1501–3.

[9] **Donlan RM, Costerton JW**. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* (2002); 15(2):167–93.

[10] **Harriott MM, Lilly EA, Rodriguez TE, et al.** *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology* (2010); 156(Pt 12):3635–44.

[11] **Chassot F, Negri MFN, Svidzinski AE, et al.** Can intrauterine contraceptive devices be a *Candida albicans* reservoir? *Contraception* (2008); 77(5):355–339.

[12] **Lal P, Agarwal V, Pruthi P, et al.** Biofilm formation by *Candida albicans* isolated from intrauterine devices. *Indian J Microbiol* (2008); 48(4):438–44.

[13] **Špaček J, Buchta V, Jílek P, et al.** Clinical aspects and luteal phase assessment in patients with recurrent vulvovaginal candidiasis. *Eur J Obstet Gynecol Reprod Biol* (2007); 131(2):198–202.

[14] **Dennerstein GJ, Ellis DH**. Oestrogen, glycogen and vaginal candidiasis. *Aust New Zeal J Obstet Gynaecol* (2001); 41(3):326–8.

[15] **Keller MJ, Guzman E, Hazrati E, et al.** PRO 2000 elicits a decline in genital tract immune mediators without compromising intrinsic antimicrobial activity. *AIDS* (2007); 21(4):467–76.

[16] **Malloy PJ, Zhao X, Madani ND, et al.** Cloning and expression of the gene from *Candida albicans* that encodes a high-affinity corticosteroid-binding protein. *Proc Natl Acad Sci* (1993); 90(5):1902–6.

[17] **Banerjee D, Martin N, Nandi S, et al.** A genome-wide steroid response study of the major human fungal pathogen *Candida albicans*. *Mycopathologia* (2007); 164(1):1–17.

[18] **Alves CT, Silva S, Pereira L, et al.** Effect of progesterone on *Candida albicans* vaginal pathogenicity. *Int J Med Microbiol* (2014); 304(8):1011–7.

[19] **Noble SM, Johnson AD**. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell* (2005); 4(2):298–309.

[20] **Znaidi S, Nesseir A, Chauvel M, et al.** A comprehensive functional portrait of two heat shock factor-type transcriptional regulators involved in *Candida albicans* morphogenesis and virulence. *PLoS Pathog* (2013); 9(8):e1003519.

[21] **Silva S, Henriques M, Martins A, et al.** Biofilms of non- *Candida albicans Candida* species: quantification, structure and matrix composition. *Med Mycol* (2009); 47(7):681–9.

[22] **Silva S, Henriques M, Oliveira R, et al.** *In vitro* biofilm activity of non-*Candida albicans Candida* species. *Curr Microbiol* (2010); 61(6):534–40.

[23] **Hawser S**. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronised *C. albicans* cells. *Med Mycol* (1996); 34(2):149–52.

[24] **DuBois M, Gilles KA, Hamilton JK, et al.** Colorimetric method for determination of sugars and related substances. *Anal Chem* (1956); 28(3):350–6.

[25] **Rodrigues CF, Gonçalves B, Rodrigues ME, et al.** The effectiveness of voriconazole in therapy of *Candida glabrata*'s biofilms oral infections and its influence on the matrix composition and gene expression. *Mycopathologia* (2017).

[26] **CLSI**. *Reference method for broth dilution antifungal susceptibility testing of yeasts*. 4th ed. CLSI standard M27. Wayne, PA: Clinical and Laboratory Standards Institute; (2017).

[27] **Bernardo RT, Cunha D V., Wang C, et al.** The CgHaa1-regulon mediates response and tolerance to acetic acid stress in the human pathogen *Candida glabrata*. *Genes | Genomes | Genetics* (2017); 7(1):1–18.

[28] **Rossignol T, Logue ME, Reynolds K, et al.** Transcriptional response of *Candida parapsilosis* following exposure to farnesol. *Antimicrob Agents Chemother* (2007); 51(7):2304–12.

[29] **Skrzypek MS, Arnaud MB, Costanzo MC, et al.** New tools at the *Candida* Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res* (2010); 38(Database issue):D428-432.

[30] **Altschul S, Madden TL, Schäffer AA, et al.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* (1997); 25(17):3389–402.

[31] **Livak KJ, Schmittgen TD**. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* (2001); 25(4):402–8.

[32] **Boskey ER**. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Hum Reprod* (2001); 16(9):1809–13.

[33] **Al-Fattani MA, Douglas LJ**. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J Med Microbiol* (2006); 55(Pt 8):999–1008.

[34] **Monteiro DR, Silva S, Negri M, et al.** Silver colloidal nanoparticles: effect on matrix composition and structure of *Candida albicans* and *Candida glabrata* biofilms. *J Appl Microbiol* (2013); 114(4):1175–83.

[35] **Ferreira C, Gonçalves B, Vilas Boas D, et al.** *Candida tropicalis* biofilm and human epithelium invasion is highly influenced by environmental pH. *Pathog Dis* (2016); 74(8):ftw101.

[36] **García-Sánchez S, Aubert S, Iraqui I, et al.** *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot Cell* (2004); 3(2):536–45.

[37] **Murillo LA, Newport G, Lan C-Y, et al.** Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot Cell* (2005); 4(9):1562–73.

[38] **Yeater KM, Chandra J, Cheng G, et al.** Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* (2007); 153(8):2373–85.

[39] **Desai J V, Bruno VM, Ganguly S, et al.** Regulatory role of glycerol in *Candida albicans* biofilm formation. *MBio* (2013); 4(2):e00637-12.

[40] **Nett JE, Lepak AJ, Marchillo K, et al.** Time course global gene expression analysis of an *in vivo Candida* biofilm. *J Infect Dis* (2009); 200(2):307–13.

[41] **Nobile CJ, Fox EP, Nett JE, et al.** A recently evolved transcriptional network controls biofilm development in *Candida albicans*. *Cell* (2012); 148(1–2):126–38.

[42] **Yi S, Sahni N, Daniels KJ, et al.** Self-induction of a/a or alpha/alpha biofilms in *Candida albicans* is a pheromone-based paracrine system requiring switching. *Eukaryot Cell* (2011); 10(6):753–60.

[43] **Garcia MC, Lee JT, Ramsook CB, et al.** A role for amyloid in cell aggregation and biofilm formation. *PLoS One* (2011); 6(3):e17632.

[44] **Srikantha T, Daniels KJ, Pujol C, et al.** Identification of genes upregulated by the transcription factor Bcr1 that

are involved in impermeability, impenetrability, and drug resistance of *Candida albicans* a/α biofilms. *Eukaryot Cell* (2013); 12(6):875–88.

[45] **Bonhomme J, Chauvel M, Goyard S, et al.** Contribution of the glycolytic flux and hypoxia adaptation to efficient biofilm formation by *Candida albicans*. *Mol Microbiol* (2011); 80(4):995–1013.

[46] **Monteiro PT, Pais P, Costa C, et al.** The PathoYeastract database: an information system for the analysis of gene and genomic transcription regulation in pathogenic yeasts. *Nucleic Acids Res* (2017); 45(D1):D597-603.

[47] **McCall AD, Kumar R, Edgerton M**. *Candida albicans* Sfl1/Sfl2 regulatory network drives the formation of pathogenic microcolonies. *PLoS Pathog* (2018); 14(9):e1007316.

[48] **Bauer J, Wendland J**. *Candida albicans* Sfl1 suppresses flocculation and filamentation. *Eukaryot Cell* (2007); 6(10):1736–44.

[49] **Conlan RS, Tzamarias D**. Sfl1 functions via the co-repressor Ssn6-Tup1 and the cAMP-dependent protein kinase Tpk2. *J Mol Biol* (2001); 309(5):1007–15.

[50] **Karnani N, Gaur NA, Jha S, et al.** *SRE1* and *SRE2* are two specific steroid-responsive modules of Candida drug resistance gene 1 (*CDR1*) promoter. *Yeast* (2004); 21(3):219–39.

[51] **Larsen B, Anderson S, Brockman A, et al.** Key physiological differences in *Candida albicans* CDR1 induction by steroid hormones and antifungal drugs. *Yeast* (2006); 23(11):795–802.

[52] **Singh A, Mahto KK, Prasad R**. Lipidomics and *in vitro* azole resistance in *Candida albicans*. *Omi A J Integr Biol* (2013); 17(2):84–93.

[53] **Finkel JS, Xu W, Huang D, et al.** Portrait of *Candida albicans* adherence regulators. *PLoS Pathog* (2012); 8(2):e1002525.

[54] **Askew C, Sellam A, Epp E, et al.** The zinc cluster transcription factor Ahr1p directs Mcm1p regulation of *Candida albicans* adhesion. *Mol Microbiol* (2011); 79(4):940–53.

[55] **Nobile CJ, Nett JE, Hernday AD, et al.** Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol* (2009); 7(6):e1000133.

[56] **Fiori A, Kucharikova S, Govaert G, et al.** The heat-induced molecular disaggregase Hsp104 of *Candida albicans* plays a role in biofilm formation and pathogenicity in a worm infection model. *Eukaryot Cell* (2012); 11(8):1012–20.

[57] **Sahni N, Yi S, Daniels KJ, et al.** Genes selectively up-regulated by pheromone in white cells are involved in biofilm formation in *Candida albicans*. *PLoS Pathog* (2009); 5(10):e1000601.

Chapter 6

General conclusions and work perspectives

6.1 General conclusions

Changes in the vaginal environment are generally required for the alteration of the opportunistic *Candida* organisms from commensal to pathogenic, promoting VVC. Having a risk factor for VVC increases the chance of getting the infection but does not always lead to it. Also, the absence of any known risk factor does not necessarily avoid VVC development. Importantly, *Candida* species contribute to the disease process using virulence factors that promote host cell damage and microbiological failure. Despite research advances, there are still a number of mechanisms involved in VVC development that need to be clarified. As a consequence, this work intended to increase the knowledge on *C. albicans* and *C. glabrata* vaginal virulence, by studying the modulation of relevant their virulence factors by vaginal conditions and identifying potential vaginal virulence determinants.

VVC usually develops at normal acidic conditions but it may also occur at situations that lead to temporary or permanent elevated vaginal pH. In this investigation, all vaginal isolates of *C. albicans* and *C. glabrata* studied were able to growth and display relevant virulence features in either acidic and neutral conditions, but presented species-specific differences in the response to pH. *Candida glabrata* strains presented increased ability to produce biofilms at acidic conditions, promoted by lactic acid, suggesting high adaptability to the normal vaginal environment. In contrast, *C. albicans* strains presented higher filamentation and biofilm quantity at neutral conditions, also suggesting *C. albicans* biofilm dispersion at acidic conditions, what may contribute to the dissemination of an infection. Additionally, strain-dependency was observed in the pH influence on biofilm structure and matrix composition. Different pH responses between *Candida* species and strains, makes them challenging pathogens in the vaginal environment, increasing the difficulty to prevent and treat VVC, especially in presence of mixed species.

In order to deep the knowledge on *C. glabrata* biofilm matrix production at acidic conditions, its composition and regulation, specifically by the transcription factor Zap1, was studied. Zap1 was found to be a negative regulator of matrix production, namely of carbohydrate, (1,3)-β-D-glucan and protein quantities. Interestingly, the results suggest that the negative regulation of matrix carbohydrate content by Zap1 has a potential relation with its blockade of the secretion of proteins with a putative role in carbohydrate matrix delivery and organization. Furthermore, the acidic conditions were found to specifically modulate the matrix proteomic profile, here revealed for the first time in *C. glabrata*, and Zap1 is suggested to intermediate this modulation, through the induction of the secretion of several

stress-response proteins. This study reveals some mechanisms underlying *C. glabrata* biofilm matrix regulation at acidic conditions, disclosing several potential vaginal virulence determinants.

It is known that VVC is promoted by conditions that increase the reproductive hormones levels, as pregnancy. In this investigation progesterone and β-estradiol were found to modulate defence and virulence traits of *C. albicans* and *C. glabrata* vaginal isolates, in a dose-dependent and species-specific fashion. The results suggest that *C. glabrata* biofilms have high adaption to hormonal stress at acidic conditions, but the hormones affect *C. albicans* filamentation and potentially increase its biofilm dispersion. Additionally, hormones were found to reduce the susceptibility of both species to oxidative stress and azoles, with clinical significance in the presence of pregnancy hormonal levels. These results suggest that hormones may act as environmental cues that promote *in vivo* survival of *Candida* species, contributing to their adaption to the vaginal environment and protecting against harmful conditions. These hormonal effects may have implications in the development and treatment of VVC, especially in risk groups presenting high hormonal levels, as pregnant women.

In order to gain further insights into the molecular mechanisms by which progesterone, at pregnancy level, affects the physiology and response of *C. albicans* biofilms, its modulation of the acidic biofilm transcriptome was assessed. Consistent with the reduced biofilm amount obtained in presence of progesterone, this hormone was shown to repress the expression of several biofilm regulators, including Tec1 and many of its target genes, suggesting that the function of this transcription factor is inhibited in the presence of the progesterone. Furthermore, the hormone was shown to induce the expression of efflux pumps, consistent with the decreased susceptibility of *C. albicans* biofilm cells to fluconazole. The acidic conditions alone were also found to modulate the transcriptomic profile of *C. albicans* biofilms, which was here reported for the first time at acidic pH. Numerous genes specifically regulated by the acidic environment in the biofilm mode were revealed, which, along with their regulators, represent an interesting cohort to search for novel players involved in biofilm formation in the acidic vaginal tract. Importantly, Sfl1 was found to be a relevant regulator of gene expression at acidic conditions and was shown to be essential for maximal biofilm formation. This study shows that the complex regulatory networks governing biofilm formation in *C. albicans* is greatly shaped by vaginal environmental conditions.

In summary, this investigation reveals that vaginal conditions specifically modulate *C. albicans* and *C. glabrata* virulence traits and associated determinants. As such, the identification of species-specific virulence determinants that may settle the ability of *Candida* species to survive in the vaginal environment may contribute to the disclosure of new targets for novel therapeutics to treat VVC.

6.2 Work perspectives

The work described in this thesis provided a useful insight into several aspects of *C. albicans and C. glabrata* vaginal virulence, however only a small fraction of this knowledge was achieved. In fact, numerous mechanisms underlying the VVC development were not investigated in this investigation, which also leads to interesting new questions for further research. Some studies suggested for future investigations are:

a) To extend the studies performed with vaginal isolates in this investigation to additional strains, preferentially clinically characterized, in order to confirm the results observed and to obtain further insights into strain-dependent modulation. Specifically, the use of vaginal strains isolated from different clinical situations, as women colonized and infected, as well as presenting known risk factors, as pregnancy, diabetes, immunossupression, and antibiotic use, will contribute to a deeper analysis of *Candid*a vaginal virulence traits and the influence of the clinical situation on them. For instance, the use of vaginal isolates from women with different hormonal situations, as in different stages of the menstrual cycle or pregnancy, as well as pre-menstrual and menopausal situations, will be interesting in the studies of the influence of hormones performed in this investigation.
b) To deep the analysis of the influence of environmental vaginal conditions on *Candida* virulence traits by analysing other relevant conditions, including glucose concentration, presence/absence of lactobacillus and of components secreted by the host immune system of externally acquired, as semen or condom-related. Additionally, the use of synthetic vaginal fluid will be valuable to mimic the vaginal environment as wells the use of the materials of uterine devices to perform biofilm-related studies.
c) To perform studies related with vaginal virulence in other *Candida* species found in the vaginal tract than *C. albicans* and *C. glabrata*, including *C. parapsiolosis*, *C. tropicalis* and *C. krusei*. Additionally, the evaluation of the co-infection process in the vaginal environment will be valuable, crossing *Candida* species and also vaginal bacterial pathogens, since mixed vaginal infections can occur and are a high challenge to manage.
d) To extend the *in vitro* studies into *ex vivo* and *in vivo* investigations, using reconstituted human vaginal epithelium (RHVE) and mouse-models, respectively. These studies allow the additional analysis of realistic *Candida*-host interactions, which can specifically affect the mechanisms underlying *Candida* vaginal virulence.

e) To further investigate the role and relevance of the several potential vaginal virulence determinants revealed in this investigation, which could lead to the identification of new targets to treat VVC. Additionally, numberless potential *Candida* vaginal virulence determinants could still be identified using global proteomic, transcriptomic and genomic approaches. In this investigation, microarrays analysis and liquid chromatography-tandem mass spectrometry, were found to be useful to perform large scale identification of potential virulence determinants, but other high-throughput techniques will be also interesting, as RNA-Seq, which allows the analysis of clinical isolates, mixed cultures and host cells response. Furthermore, the combined use of gene deletion techniques to perform omics analysis in mutant strains will be valuable to decipher the functional role of potential virulence determinants.

Annex I

Table AI.1 Primers used in the study of Chapter 3

Primer	Sequence (5´→ 3´)	Description
ACT1_Fw	GACGCTCAGTGCACACAACT	To amplify *ACT1* gene
ACT1_Rv	GCAAAACCGGCTTTACACAT	
ZAP1_Fw	TTGATGTGTTTGGCCATGGG	To amplify *ZAP1* gene
ZAP1_Rv	ACAACAACCGCATCCACTTC	
ZAP1_before_Fw	AGCTGGGCCCGCAAGAGGGACAAATCGTCGG	To amplify the upstream flanking sequence of *ZAP1* gene
ZAP1_before_Rv	AGCTCTCGAGCGTTATCCTCTTGTACCCAAGCC	
ZAP1_after_Fw	AGCTCCGCGGCGGACACCTACTGCACCTCTG	To amplify the downstream flanking sequence of *ZAP1* gene
ZAP1_after_Rev	AGCTGAGCTCGCTCTACTCTCGCATTGATCTGC	
SAT1_Fw	GGAGCGATAAGCGTGCTTCTGC	To amplify the deletion cassette
SAT1_Rv	CCACCTGCTCAGGGATCACC	
ZAP1_P1_ Fw	CTGGAAAGTAAGAACCGCTGGC	To confirm the construction of the deletion cassette
ZAP1_P1_ Rv	GCATCAACCGGTGCTCCTGG	
ZAP1_P2_ Fw	AGGAGAAGCGATGAACCCAATTCGCCCTATAGTGAGTCG	
ZAP1_P2_ Rv	TATGCCCTTGCTTCTGGCGGCCCGGTACCCAGCTTTTG	
ZAP1_Compl_Fw	AGCTCCGCGGATGGTGGAAGGAGTAGTGCACG	To amplify the flanking sequence of *ZAP1* plus the gene
ZAP1_Compl_Rv	AGCTCTCGAGCTAGGTGGCAATCTGCTGGTCC	
SAT1_Compl_Fw	ACATGGTTTATGTGATCGAGGAAGTTCCTATACTTTCTAGAG	To amplify the complemented deletion cassette
SAT1_Compl_Rv	GAAATGTGTGTCTCTCTGGCGGTGGCGGCCGCTCTA	
ZAP1_P3_ Fw	GCAAGAGGGACAAATCGTCGG	To confirm the construction of the complemented deletion cassette
ZAP1_P3_ Rv	GCTCTACTCTCGCATTGATCTGC	
ZAP1_P4_ Fw	GCTTTTGGGCTCATCGCTTCGC	
ZAP1_P4_ Rv	CCACTGAAGTGCAAAGTGTGCG	

Table A1.2 List of *Candida glabrata* biofilm matrix proteins at acidic conditions. The proteins found in the matrix of *C. glabrata* ATCC 2001 biofilms developed for 24 h at pH 4 are herein shown. Proteins previously reported to be secreted by *C. glabrata* (or by *C. albicans* orthologs) are underlined and those found to be specifically secreted to the matrix at acidic conditions (Figure 2.6 – Chapter 2) are highlighted in bold. The biological function indicated is based on the information available at *Candida* Genome Database and UniProt.

Protein	Biological function
Ygk3p	Predicted kinase activity; role in response to heat, salt stress and proteolysis
Bmh1(B)p	Uncharacterized protein
Awp6p	Cell wall adhesin-like protein
Act1p	Actin
Bna3p	Putative 2-aminoadipate transaminase activity
Ugp1p	Putative glucose pyrophosphorylase involved in azole resistance
Tup1p	Protein of unknown function
Tef3p	Translation elongation factor eEF3
Sse1p	Heat shock protein of HSP70 family
Sac7p	Predicted GTPase activator activity
Pwp6p	Cell wall adhesin-like protein with similarity to flocculins; GPI anchor
Lys21p	Homocitrate synthase
Lys12p	Homo-isocitrate dehydrogenase
Kar2p	Protein with a predicted role in nuclear fusion
Ilv5p	Ketol-acid reducto-isomerase
Hxk2p	Putative hexokinase B
Gre3p	Predicted NADP oxidoreductase activity, mRNA binding activity
Gre2(A)p	Putative methylglyoxal reductase (NADPH-dependent)
Glr1p	Predicted glutathione oxidoreductase involved in oxidative stress response
Frds1p	Putative soluble fumarate reductase
Erg13p	3-hydroxy-3-methylglutaryl coenzyme A synthase
Egd2p	Alpha subunit of the polypeptide-associated complex
Cagl0m14025gp	Predicted oxidoreductase activity and role in oxidation-reduction process
Cagl0m12386gp	Predicted role in isoleucine biosynthetic process and mitochondrial translation
Cagl0m12320gp	Predicted FAD transmembrane transporter activity
Cagl0m11000gp	Ortholog(s) have cytosol localization
Cagl0m08734gp	Uncharacterized protein
Cagl0m03377gp	Predicted 1,4-alpha-glucan branching enzyme; role in glycogen biosynthesis
Cagl0m02695gp	Predicted 5S rRNA binding; role in ribosomal large subunit assembly
Cagl0m00814gp	Predicted role in maturation of SSU-rRNA
Cagl0l06908gp	Predicted ATP-dependent RNA helicase activity, RNA binding activity
Cagl0l04840gp	Putative structural constituent of ribosome activity
Cagl0l00561gp	Putative phosphatidic acid binding activity
Cagl0k12100gp	Putative coproporphyrinogen III oxidase; role in azole resistance
Cagl0k11572gp	Predicted chaperone binding activity, role in regulation of DNA binding
Cagl0k10780gp	Predicted dehydrogenase activity
Cagl0k05027gp	Predicted adenylosuccinate synthase activity
Cagl0k04235gp	Putative transaldolase
Cagl0k02123gp	Predicted role in rRNA export from nucleus, ribosome assembly
Cagl0k00913gp	Putative formate-tetrahydrofolate ligase activity,
Cagl0k00825gp	Predicted phosphatase activity and role in L-serine biosynthetic process
Cagl0j11891gp	Putative cell wall adhesin-like protein
Cagl0j08800gp	Putative inosine nucleosidase activity and hydrolase activity
Cagl0j08030gp	Putative DNA polymerase activity, single-stranded DNA binding activity
Cagl0j07568gp	Putative RNA polymerase II, cysteine-type peptidase activity
Cagl0j01331gp	Uncharacterized protein
Cagl0i01496gp	Predicted ATPase activity, enzyme regulator activity
Cagl0h10076gp	Putative plasma membrane protein
Cagl0h09856gp	Putative role in DNA methylation, cellular response to DNA damage stimulus,
Cagl0h09614gp	Putative GPI-linked cell wall protein

Cagl0h08195gp	Predicted ATPase inhibitor activity, Hsp70 and Hsp90 protein binding
Cagl0h05577gp	Predicted protein membrane anchor activity
Cagl0h03773gp	Predicted role in exonucleolytic trimming
Cagl0h02585gp	Putative glutamate decarboxylase activity, role in response to oxidative stress
Cagl0h02101gp	Predicted role in RNA metabolic process
Cagl0g08558gp	Predicted GTPase activity
Cagl0g07887gp	Predicted role in protein assembly and cell wall organization
Cagl0g07227gp	Predicted role in endonucleolytic cleavage in ITS1
Cagl0g03091gp	Predicted role in valine-tRNA ligase activity
Cagl0g02079gp	Predicted structural constituent of ribosome activity
Cagl0f07777gp	Putative aldehyde dehydrogenase; biofilm-induced
Cagl0f04213gp	Putative ATP:ADP antiporter activity
Cagl0f02937gp	Putative role in ribosomal large subunit assembly
Cagl0e06424gp	Putative role in response to oxidative stress and ergosterol biosynthetic process
Cagl0e06226gp	Predicted metalloaminopeptidase activity and serine-type peptidase activity
Cagl0e05610gp	Predicted pyruvate kinase activity, role in glycolysis and pyruvate biosynthesis
Cagl0e04752gp	Predicted proteasome binding and ubiquitin-protein transferase activity
Cagl0e02321gp	Putative phospholipase B; predicted GPI-anchor
Cagl0e02013gp	Putative cytosolic large ribosomal subunit localization
Cagl0d04752gp	Putative oxidoreductase activity and role in pyridoxal biosynthetic process
Cagl0d03432gp	Predicted ribonucleoside-diphosphate reductase activity
Cagl0d01782gp	Putative role in response to heat and pH
Cagl0d00198gp	Putative dehydrogenase activity and role in butanediol biosynthesis
Cagl0c02453gp	Putative GTPase activity
Cagl0c01243gp	Putative histidinol-phosphate transaminase activity
Cagl0c00253gp	Putative cell wall adhesin; predicted GPI anchor
Cagl0b04345gp	Predicted GTP binding activity
Cagl0a04433gp	Predicted thioredoxin peroxidase activity; role in cellular response to oxidative stress
Cagl0a03388gp	Predicted RNA binding, structural constituent of ribosome activit
Ao440_005907p*	Aspartic proteinase
Ao440_005507p*	60S ribosomal protein
Ao440_005116p*	60S ribosomal protein
Ao440_004757p*	ATP-dependent 6-phosphofructokinase
Ao440_004655p*	Elongation factor
Ao440_004202p*	60S ribosomal protein
Ao440_003817p*	ATP phosphoribosyltransferase
Ao440_003792p*	60S ribosomal protein
Ao440_003745p*	40S ribosomal protein
Ao440_003726p*	Gluconokinase
Ao440_003707p*	Catalase
Ao440_003698p*	Glycogen synthase
Ao440_003659p*	Peptidylprolyl isomerase
Ao440_003658p*	Uncharacterized protein
Ao440_003540p*	Pyruvate carboxylase
Ao440_003446p*	Phosphoribosylformylglycinamidine synthase
Ao440_003298p*	Twinfilin
Ao440_003278p*	40S ribosomal protein
Ao440_003263p*	Ubiquitin-activating enzyme
Ao440_003212p*	tRNA-aminoacylation cofactor
Ao440_003185p*	Trehalose-6-phosphate synthase
Ao440_003168p*	NADPH-dependent alpha-keto amide reductase
Ao440_003128p*	S-adenosylmethionine synthase
Ao440_003047p*	Orotate phosphoribosyltransferase
Ao440_002921p*	40S ribosomal protein
Ao440_002881p*	Mannose-6-phosphate isomerase
Ao440_002784p*	Asparagine synthetase
Ao440_002526p*	Phosphoribosylaminoimidazole-succinocarboxamide synthase
Ao440_002402p*	Eukaryotic translation initiation factor

Ao440_002328p*	Phospho-2-dehydro-3-deoxyheptonate aldolase
Ao440_002280p*	Ribosomal protein
Ao440_001844p*	60S ribosomal protein
Ao440_001734p*	Uncharacterized protein
Ao440_001659p*	Ribonucleoside-diphosphate reductase
Ao440_001557p*	60S ribosomal protein
Ao440_001527p*	60S ribosomal protein
Ao440_001491p*	Uncharacterized protein
Ao440_001488p*	Branched-chain-amino-acid aminotransferase
Ao440_001438p*	ATP-dependent 6-phosphofructokinase
Ao440_001397p*	40S ribosomal protein
Ao440_001147p*	40S ribosomal protein
Ao440_001087p*	Fatty acid synthase subunit alpha
Ao440_000932p*	Zinc-regulated transporter
Ao440_000633p*	60S ribosomal protein
Ao440_000629p*	Fatty acid synthase subunit beta
Ao440_000606p*	Farnesyl pyrophosphate synthase
Ao440_000573p*	Adenosine kinase
Ao440_000548p*	Diphosphomevalonate decarboxylase
Ao440_000539p*	Saccharopine dehydrogenase
Ao440_000495p*	Zinc-regulated protein
Ao440_000485p*	Adenosylhomocysteinase
Ao440_000435p*	Glutathione peroxidase
Ao440_000280p*	Acetyl-coenzyme A synthetase
Ao440_000249p*	Fimbrin
Ao440_000148p*	Uncharacterized protein
Yps7p	Putative aspartic protease; predicted GPI-anchor
Yps5p	Putative aspartic protease; predicted GPI-anchor
Yps3p	Putative aspartic protease; predicted GPI-anchor
Yps2p	Putative aspartic protease; predicted GPI-anchor, induced in response to low pH
Yps11p	Putative aspartic protease; predicted GPI-anchor, induced in response to low pH
Yps1p	Putative aspartic protease; predicted GPI-anchor, regulation of pH homeostasis at low pH
Vig9p	GDP-mannose pyrophosphorylase
Utr2p	Putative glycoside hydrolase; predicted GPI-anchor
Ura3p	Orotidine 5'-phosphate decarboxylase
Tsa1p	Predicted thioredoxin peroxidase involved in oxidative stress response;
Trr1p	Thioredoxin reductase (NADPH)
Tkl1p	Putative transketolase
Tir1p	Putative GPI-linked cell wall mannoprotein
Tif1p	Translation initiation factor
Tef1p	Translation elongation factor
Sun4p	Predicted role in fungal-type cell wall organization or biogenesis
Ssr1p	Putative GPI-linked cell wall protein
Ssb1p	Heat shock protein
Ssa3p	Heat shock protein of the HSP70 family
Ssa1p	Heat shock protein of the HSP70 family
Spe3p	Putative spermine/spermidine synthase
Sec14p	Predicted phosphatidylinositol binding activity
Scw4p	Putative transglycosidase with a predicted role in the modification of 1,3-beta-glucan
Scl1p	Alpha subunit of the 20S proteasome
Sam1p	S-adenosylmethionine synthetase
Rps12p	40S ribosomal protein
Rpl16Ap	Putative ribosomal protein
Rho1p	Beta-1,3-glucan synthase regulatory subunit
Pyk1p	Pyruvate kinase
Pmu2p	Putative phosphate starvation inducible acid phosphatase
Pmu1p	Protein with a phosphomutase-like domain
Pma1p	Putative plasma membrane proton pump with a predicted role in pH homeostasis

Pir5p	Pir protein family member, putative cell wall component
Pir4p	Pir protein family member, putative cell wall component
Pir3p	Pir protein family member, putative cell wall component
Pil1p	Long chain base-responsive inhibitor of protein kinase
Pgm1p	Putative phosphoglucomutase
Pfy1p	Profilin
Pdr13p	Protein with a predicted role in pleiotropic drug resistance
Pdr1p	Zinc finger transcription factor, regulates drug efflux pumps
Pdc1p	Pyruvate decarboxylase, involved in pyruvate metabolism
Mp65p	Mannoprotein
Mid1p	Putative calcium transporter
Met15p	O-acetyl homoserine sulfhydrylase (OAHSH)
Lsp1p	Long chain base-responsive inhibitor of protein kinases
Kre9p	Protein involved in cell wall beta-1,6-glucan synthesis
Hxt4p	Predicted glucose transmembrane transporter activity
Hsp31p	Putative cysteine protease
Hsp12p	Heat shock protein; upregulated in biofilm
Hsc82p	Putative heat shock protein
Hbn1p	Ortholog(s) have oxidoreductase activity
Gre2(B)p	Putative methylglyoxal reductase (NADPH-dependent)
Gpd2p	Putative glycerol 3-phosphate dehydrogenase
Gpd1p	Putative cytoplasmic glycerol-3-phosphate dehydrogenase
Gnd1p	Phosphogluconate dehydrogenase
Glk1p	Aldohexose specific glucokinase
Gcy1p	Predicted oxidoreductase activity
Gas5p	Putative 1,3-beta-glucanosyltransferase
Gas4p	Putative 1,3-beta-glucanosyltransferase
Gas2p	Putative 1,3-beta-glucanosyltransferase
Gas1p	Putative 1,3-beta-glucanosyltransferase
Fks2p	Putative 1,3-beta-glucan synthase component
Fet3p	Putative copper ferroxidase involved in iron uptake
Fba1p	Fructose-bisphosphate aldolase
Epa6p	Sub-telomerically encoded adhesin with a role in cell adhesion
Epa3p	Epithelial adhesion protein; GPI-anchored
Epa2p	Epithelial adhesion protein; predicted GPI-anchor
Eno1p	Putative enolase I
Egt2p	Putative glycoside hydrolase; predicted GPI-anchor
Eft1p	Macrophage-induced
Efb1p	Translation elongation factor
Ecm4p	Putative omega class glutathione transferase
Ecm33p	Predicted role in cell wall biogenesis and organization; predicted GPI-anchor
Dur1,2p	Predicted role in nitrogen compound metabolic process
Cwp1.2p	Putative GPI-linked cell wall protein
Cwp1.1p	GPI-linked cell wall protein
Crh1p	Putative glycoside hydrolase; predicted GPI-anchor
Cof1p	Cofilin, actin binding and severing protein
Awp12p	Adhesin-like protein
Atp1p	ATPase complex
Aro8p	Putative aromatic aminotransferase
Arg1p	Arginino succinate synthetase
Ald4p	Putative aldehyde dehydrogenase (NAD) activity
Ahp1p	Putative thiol-specific peroxiredoxin; upregulated in biofilm
Aed1p	Adhesin-like protein required for adherence to endothelial cells; predicted GPI anchor
Cagl0m05731gp	Predicted alpha-1,3-mannosyltransferase activity
Cagl0k11462gp	Predicted DNA binding activity
Cagl0h04521gp	Predicted role in translation
Cagl0m14003gp	Predicted role in proteasomal ubiquitin-independent protein catabolic process
Cagl0m13651gp	Predicted carboxypeptidase activity

Cagl0m13629gp	Uncharacterized protein
Cagl0m11242gp	Predicted carbonyl reductase (NADPH) activity
Cagl0m10241gp	Predicted RNA binding activity, role in macroautophagy
Cagl0m09889gp	Uncharacterized protein
Cagl0m08756gp	Putative exo-1,3-beta-glucanase; predicted GPI-anchor
Cagl0m05599gp	Predicted cell surface, extracellular region and cell wall localization
Cagl0m04543gp	Protein of unknown function
Cagl0m03861gp	Predicted nucleotide binding, structural constituent of ribosome activity
Cagl0m02343gp	Predicted ligase activity
Cagl0l12694gp	Predicted phosphatidylinositol binding activity
Cagl0l12144gp	Predicted role in actin filament bundle organization
Cagl0l11836gp	Predicted role in mitotic cell cycle
Cagl0l08294gp	Predicted role in axial cellular bud site selection and cell division site
Cagl0l07502gp	Putative GPI-linked cell wall protein
Cagl0l06424gp	Predicted GPI-linked adhesin-like protein
Cagl0l03289gp	Putative secreted beta-glucosidase
Cagl0l00407gp	Predicted ATP-dependent DNA helicase activity
Cagl0k12672gp	Predicted GTPase activity
Cagl0k07414gp	Predicted cytosolic large ribosomal subunit localization
Cagl0k06149gp	Predicted role in ribosomal small subunit assembly
Cagl0k05137gp	Predicted alpha-trehalase activity and role in cellular response to ethanol
Cagl0k03289gp	Glucose-6-phosphate epimerase
Cagl0k03135gp	Predicted role in maturation of SSU-rRNA from tricistronic rRNA
Cagl0k01353gp	Predicted role in intracellular sterol transport and extracellular localization
Cagl0k00935gp	Putative phosphatase activity
Cagl0j11220gp	Predicted DNA-endonuclease activity
Cagl0j10560gp	Predicted role in attachment of spindle microtubules to kinetochore
Cagl0j09922gp	Predicted glucan endo-1,3-beta-D-glucosidase activity
Cagl0j09086gp	Predicted role in maturation of LSU-rRNA from tricistronic rRNA transcript
Cagl0j09064gp	Predicted GTPase activity, role in Golgi to plasma membrane transport
Cagl0j07128gp	Putative extracellular region localization
Cagl0j06050gp	Predicted role in cell wall assembly and extracellular region localization
Cagl0j04752gp	Predicted role in cell wall organization
Cagl0j04466gp	Predicted role in cell wall organization
Cagl0i08943gp	Predicted nucleic acid binding, nucleotide binding activity
Cagl0i08833gp	Predicted role in clathrin-dependent endocytosis
Cagl0i05874gp	Haloacid dehalogenase-like hydrolase
Cagl0i00594gp	Putative GTPase activity
Cagl0i00462gp	Uncharacterized protein
Cagl0h08844gp	Putative stress protein
Cagl0h07887gp	Putative phosphoribosylamine-glycine ligase activity
Cagl0h03971gp	Predicted role in cellular response to oxidative stress
Cagl0h03707gp	Predicted misfolded protein binding activity
Cagl0h00462gp	Predicted structural constituent of ribosome activity
Cagl0g09845gp	Predicted role in snoRNA splicing
Cagl0g08668gp	Predicted role in cell wall organization
Cagl0g08173gp	Predicted structural constituent of ribosome activity
Cagl0g07623gp	Putative aminopeptidase activity, role in protein catabolic process in the extracellular region
Cagl0g06358gp	Predicted role in Golgi to plasma membrane transport
Cagl0g05357gp	Protein of unknown function
Cagl0g05027gp	Putative structural constituent of ribosome activity
Cagl0g01738gp	Protein of unknown function
Cagl0f08833gpp	Putative adhesin-like protein
Cagl0f05137gp	Predicted cholesterol binding, sterol binding activity, extracellular region localization
Cagl0f05115gp	Predicted plasma membrane localization
Cagl0f04895gp	Predicted glycogen phosphorylase activity
Cagl0f04873gp	Predicted glucosidase activity and role in (1,6)-beta-D-glucan biosynthetic process
Cagl0f03003gp	Putative osmosensor activity and role in (1,3)-beta-D-glucan biosynthetic process

Cagl0f02123gp	Predicted GTPase activity
Cagl0f01749gp	Putative serine hydroxymethyltransferase
Cagl0f00759gp	Putative mannosyltransferase
Cagl0f00737gp	Predicted ubiquitin protein ligase binding activity
Cagl0e06600gp	Putative adhesin-like protein
Cagl0e03828gp	Predicted molecular adaptor activity
Asc1p	40S small subunit ribosomal protein
Cagl0d01012gp	Predicted DNA-binding transcription activator activity
Cagl0d00418gp	Predicted role in long-chain fatty acid transport
Cagl0c04323gp	Predicted role in trehalose catabolic process
Cagl0c03916gp	Predicted transferase activity, role in protein glycosylation
Cagl0c03113gp	Putative hydrolase activity, acting on acid anhydrides
Cagl0b04257gp	Putative cell surface, cytosolic large ribosomal subunit
Cagl0b03685gp	Putative flavodoxin
Cagl0b03619gpp	Predicted endopeptidase activity
Cagl0b01100gp	Phosphatidylethanolamine-binding protein
Cagl0a04829gp	Putative hexokinase isoenzyme
Cagl0a03366gp	Predicted cyclo hydrolase activity
Cagl0a02882gp	Protein of unknown function
Cagl0a02299gp	Protein of unknown function
Cagl0a02255gp	Protein of unknown function
Cagl0a01672gp	Protein of unknown function
Cagl0a01474gp	Predicted cell surface, extracellular region and cell wall localization
Cagl0a00561gp	Predicted role in proteasome regulatory particle assembly
Ao440_005908p*	Predicted aspartyl protease
Ao440_005893p*	Uncharacterized protein
Ao440_005882p*	40S ribosomal protein
Ao440_005633p*	Putative cystathionine beta-synthase
Ao440_005468p*	Putative aspartic-type endopeptidase activity
Ao440_005420p*	Uncharacterized protein
Ao440_005150p*	Predicted Phosphoglycerate kinase
Ao440_004901p*	Predicted Lysophospholipase
Ao440_004850p*	Predicted translationally-controlled tumor protein-like protein
Ao440_004828p*	Putative acetyl-CoA acetyltransferase
Ao440_004788p*	60S ribosomal protein
Ao440_004657p*	Predicted malate dehydrogenase
Ao440_004628p*	40S ribosomal protein
Ao440_004403p*	Putative phosphoglycerate dehydrogenase
Ao440_004354p*	Predicted midasin
Ao440_004274p*	Predicted endochitinase
Ao440_004205p*	Putative cell wall protein
Ao440_004073p*	Predicted mannan endo-1,6-alpha-mannosidase
Ao440_003976p*	Predicted histone
Ao440_003952p*	Putative saccharopepsin
Ao440_003895p*	Putative ornithine aminotransferase
Ao440_003870p*	Putative homoserine dehydrogenase
Ao440_003863p*	Predicted branched-chain-amino-acid aminotransferase
Ao440_003848p*	Putative mannan endo-1,6-alpha-mannosidase
Ao440_003806p*	Predicted glutamate dehydrogenase
Ao440_003801p*	Uncharacterized protein
Ao440_003793p*	Predicted phosphomannomutase
Ao440_003750p*	Predicted protoplast secreted protein
Ao440_003643p*	Predicted threonine synthase
Ao440_003531p*	60S ribosomal protein
Ao440_003518p*	Putative guanylate kinase
Ao440_003429p*	Putative acyl-CoA-binding protein
Ao440_003398p*	Putative phosphoglucomutase 2
Ao440_003284p*	Putative thioredoxin

Ao440_003257p*	Putative cell wall protein
Ao440_003211p*	60S ribosomal protein
Ao440_003092p*	Predicted glucose-6-phosphate 1-dehydrogenase
Ao440_002855p*	Putative cell wall protein
Ao440_002798p*	Uncharacterized protein
Ao440_002673p*	Predicted alcohol dehydrogenase
Ao440_002603p*	Predicted cell wall mannoprotein
Ao440_002550p*	Predicted methyltransferase
Ao440_002449p*	Predicted NADPH dehydrogenase
Ao440_002445p*	Predicted enolase
Ao440_002432p*	Predicted 6-phosphogluconolactonase-like protein
Ao440_002401p*	Putative cytochrome c
Ao440_002376p*	40S ribosomal protein S16
Ao440_002362p*	Putative glucan 1,3-beta-glucosidase
Ao440_002323p*	Putative protoplast secreted protein
Ao440_002317p*	Predicted inorganic pyrophosphatase
Ao440_002262p*	Predicted non-histone chromosomal protein
Ao440_002252p*	Predicted triosephosphate isomerase
Ao440_002164p*	Predicted cystathionine gamma-lyase
Ao440_002134p*	40S ribosomal protein S6
Ao440_002126p*	Putative glucose-6-phosphate isomerase
Ao440_002105p*	Putative mannose-1-phosphate guanyltransferase 2
Ao440_002010p*	Putative glycogenin
Ao440_001920p*	Putative ATP synthase subunit beta
Ao440_001875p*	Putative glucan 1,3-beta-glucosidase
Ao440_001854p*	Putative membrane protein
Ao440_001754p*	40S ribosomal protein S7
Ao440_001725p*	Predicted topoisomerase
Ao440_001634p*	60S ribosomal protein
Ao440_001523p*	60S acidic ribosomal protein
Ao440_001494p*	Predicted glucan 1,3-beta-glucosidase
Ao440_001295p*	Putative glycogen synthase
Ao440_001110p*	Putative cell wall protein
Ao440_001097p*	Putative phosphoglycerate mutase
Ao440_001018p*	Uncharacterized protein
Ao440_001016p*	Predicted cell wall protein
Ao440_000985p*	60S ribosomal protein L
Ao440_000924p*	Predicted peptidyl-prolyl cis-trans isomerase
Ao440_000923p*	Predicted aspartate-semialdehyde dehydrogenase
Ao440_000887p*	Uncharacterized protein
Ao440_000698p*	Putative guanine nucleotide-binding protein subunit
Ao440_000688p*	Putative aspartate aminotransferase
Ao440_000603p*	Putative flocculation protein
Ao440_000457p*	Putative histone
Ao440_000447p*	Putative histone
Ao440_000355p*	Putative ras-like protein
Ao440_000292p*	Putative transaldolase
Ao440_000222p*	Predicted 6,7-dimethyl-8-ribityllumazine synthase
Ao440_000151p*	Predicted disulfide-isomerase
Ao440_000149p*	Putative cell wall protein
Ao440_000147p*	Predicted phosphotransferase
Ao440_000135p*	40S ribosomal protein
Ao440_000093p*	Predicted thioredoxin reductase
Ao440_000070p*	Putative sortilin
Ao440_000056p*	60S ribosomal protein
Ao440_000055p*	Putative glucosidase
Ao440_000005p*	Predicted phosphatidylinositol 3-phosphate-binding protein

Table A1.3 List of *Candida glabrata* biofilm matrix proteins found to be regulated by *Zap1*. The proteins found to be specifically present in the matrix of *zap1Δ* biofilms developed for 24 h at pH 4, comparative to the matrix of z*ap1Δ*:: *ZAP1* biofilms are herein shown. The biological function indicated is based on the information available at *Candida* Genome Database.

Protein	Biological function
Pwp3p	Putative adhesin-like protein
Awp7p	Putative adhesin-like cell wall protein; predicted GPI-anchor
Pir1p	Putative cell wall component, Pir protein family member
Epa22p	Putative adhesin-like protein
Cox12p	Cytochrome c oxidase
Ckb2p	Predicted protein kinase regulator activity
Cagl0m13211gp	Uncharacterized protein
Cagl0m11726gp	Putative GPI-linked cell wall adhesin-like protein
Cagl0m03773gp	Predicted GPI-linked adhesin-like protein
Cagl0l13200gp	Predicted role in endoplasmic reticulum to Golgi vesicle-mediated transport
Cagl0l10670gp	Predicted GPI-linked cell wall protein, involvement in cell wall 1,6-beta-glucan biosynthesis
Cagl0l09911gp	Putative adhesin-like cell wall protein; predicted GPI-anchor
Cagl0l07128gp	Putative clathrin binding activity
Cagl0k12892gp	Putative GTPase activator activity
Cagl0i00506gp	Putative glutathione hydrolase activity, role in xenobiotic metabolic process
Cagl0h06611gp	Uncharacterized protein
Cagl0h03993gp	Putative citrate synthase activity, role in acetyl-CoA catabolic process
Cagl0g05632gp	Uncharacterized protein
Cagl0d05742gp	Predicted glycotransferase activity, role in protein glycosylation
Cagl0d02222gp	Putative role in mitochondrial mRNA processing
Cagl0c05533gp	Uncharacterized protein
Ao440_005873p*	Aspartic proteinase
Ao440_003803p*	Hyphally regulated cell wall protein
Ao440_003501p*	Glutaredoxin
Ao440_003410p*	Uncharacterized protein
Ao440_002768p*	Flocculation protein
Ao440_002189p*	Endoplasmic oxidoreductin
Ao440_001871p*	Glyceraldehyde-3-phosphate dehydrogenase
Ao440_001810p*	Assembly protein
Ao440_001780p*	Ribosomal protein
Ao440_001107p*	Flocculation protein

Annex II

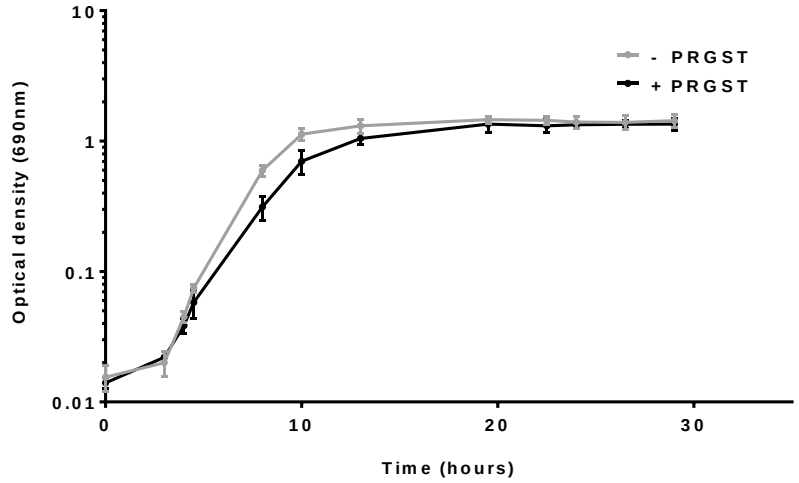


Figure AII.1 Progesterone effect on *Candida albicans* planktonic cells. Planktonic growth curves of *C. albicans* SC5314 cells cultivated in RPMI at pH 4 in the absence (-PRGST) or presence of 2 μM of progesterone (+PRGST).

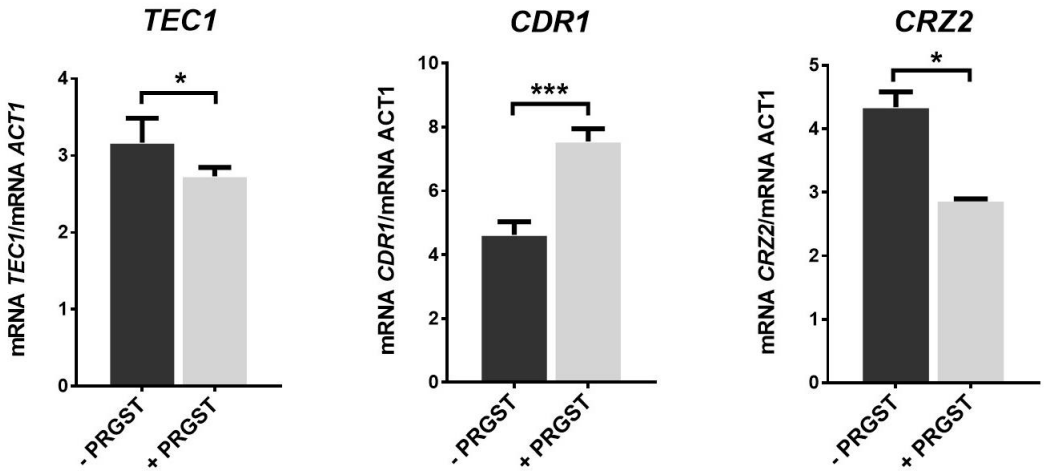


Figure AII.2 Transcript levels, estimated by qRT-PCR, of *Candida albicans TEC1*, *CDR1* and *CZR2* genes. Are presented the levels produced in *C. albicans* SC5314 biofilms cells cultivated 24 h in RPMI at pH 4 in the absence (-PRGST) or presence of 2 μM of progesterone (+PRGST). The values of the transcript levels were normalized using as internal control the levels of ACT1 mRNA. Error bars represent standard deviation. Asterisks represent statistical difference between the conditions (*P-value ≤0.05; ***P-value ≤0.0001).

Annex III

Table AII.1 Forward (FW) and reverse (RV) primers used for qRT-PCR of Chapter 5.

Target gene /Name of the ORF	**Sequence (5´→ 3´)**	**Orientation**
ACT1 / C1_13700W_A	GACGCTCAGTGCACACAACT	FW
	GCAAAACCGGCTTTACACAT	RV
NDT80/C2_00140W_A	TCAGCAACAACTGCCTCAAC	FW
	TGGTCGACGATTCCAACATA	RV
WOR1/ C1_10150W_A	CCACCAGCAGTCAGTACCAA	FW
	TAGTCATTGGCATGGGTTCA	RV
CSA2/ C4_06920C_A	ACCCTCCTGTTCCCAAAACT	FW
	ACGACATTTGGATGCAACAC	RV
NPL3/ C1_14280C_A	GAGCTGCACCTGTTGTTGAA	FW
	CCTTCTGCCAAGTTGGTGAT	RV
PBR1/ C1_06370C_A	TGCTGCTGGTTCTGATGGTA	FW
	CTTTGGTGGCAGATTTGGAT	RV
AHR1/ C3_06000W_A	GGTCGCAATTGAAAAATGCT	FW
	GGGAAAACAATTTGGCTTGA	RV
HYR1/C1_13450W_A	GGTTCTGGCTCTCAAACTGG	FW
	CCACTTCCAGAACCGTTGTT	RV
TPK1/C1_10220C_A	CCAACGATTCCCTACTCCAG	FW
	TTTTGTTGCCACCACTTCAG	RV
IFE2/CR_05340C_A	GGAAACAAGGCCACTTTCAA	FW
	TGCCTTGACAACCAATTCAA	RV
QDR1/CR_04210C_A	GGTTGGTGCAGTTTCTGGTT	FW
	TATGGACCCATTCCCCACTA	RV
C7_04080C_A	GGTTGGTGCAGTTTCTGGTT	FW
	TATGGACCCATTCCCCACTA	RV
C7_01510W_A	ATTCTTTTGGGGGATTGGTC	FW
	ATGGCCAAAACAATTGATCC	RV
TEC1/ C3_04530C_A	ACACTTGCAACCACACCAAA	FW
	TGCGTGTAGGAAACACCAAA	RV
CDR1 / C3_05220W_A	CCAATCCAGCTGAAAGAGAA	FW
	TAAGTTTCCCTGGTGTTGGA	RW
CRZ2/ CR_07060C_A	ACTTCTTCAGCCACGTCATC	FW
	TTATCATGCAGTCGAGCAAA	RW

Table AIII.2 List of genes found to be biofilm-regulated (up- and down) in *Candida albicans* cells cultivated 24 h at pH 4. Genes whose expression was found to increase or decrease (above or below 2-fold) in *C. albicans* SC5314 biofilms grown 24 h in RPMI at pH 4 in comparison with the transcript levels registered in planktonic cells cultivated in the same conditions, were selected and are herein shown. Genes whose transcription was found to be biofilm-induced or -repressed specifically at the acidic conditions used in this study are highlighted in grey and among them are highlighted in bold those previously described as essential to *C. albicans* biofilm formation. The biological function indicated is based on the information available at Candida Genome Database.

Up-regulated genes

Gene	Biological function	$\frac{\text{mRNA}_{\text{biofilm}}}{\text{mRNA}_{\text{planktonic}}}$
INO1	Inositol-1-phosphate synthase	28.05
ALS1	Cell-surface adhesin	20.17
ECE1	Candidalysin	18.12
CAN2	Basic amino acid permease	16.91
SOD5	Cu-containing superoxide dismutase	15.01
GAP1	Amino acid permease	14.33
C1_13130C_A	Putative histidine permease	12.59
ENA2	Putative sodium transporter	10.99
COX3B	Cytochrome c oxidase involved in mitochondrial respiration	10.97
HGT2	Putative MFS glucose transporter	10.57
MRV8	Unknown function	10.56
MUP1	Putative high affinity methionine permease	10.07
HYR1	Hyphal cell wall protein involved in biological adhesion	9.87
NAD1	NADH dehydrogenase involved in mitochondrial respiration	9.69
PGA45	Putative GPI-anchored cell wall protein	9.66
NAD3	NADH dehydrogenase involved in mitochondrial respiration	9.49
C2_10160W_A	Secreted protein	9.30
HGT1	High-affinity MFS glucose transporter	9.18
DIP5	Dicarboxylic amino acid permease	8.97
NAD5	NADH dehydrogenase involved in mitochondrial respiration	8.59
SKN1	Protein with a role in beta-1.6-glucan synthesis	8.58
COX2	cytochrome c oxidase involved in mitochondrial respiration	8.56
NAD6	NADH dehydrogenase involved in mitochondrial respiration	8.54
ALS2	ALS family protein	8.42
PHR1	Cell surface glycosidase	8.33
NAD4	NADH dehydrogenase involved in mitochondrial respiration	8.30
SIT1	Transporter of ferrichrome siderophores	8.12
IHD1	GPI-anchored protein	7.94
ATP6	Subunit 6 of the F0 sector of mitochondrial F1F0 ATP synthase	7.84
NAD2	NADH dehydrogenase involved in mitochondrial respiration	7.81
C1_01510W_A	Unknown function	7.57
HMX1	Heme oxygenase	7.53
PHO84	High-affinity phosphate transporter	7.41
GDH2	Putative NAD-specific glutamate dehydrogenase	7.06
NAD4L	NADH dehydrogenase involved in mitochondrial respiration	7.05
HIP1	Amino acid permease	6.91
ATP9	Subunit 9 of the F0 sector of mitochondrial F1F0 ATP synthase	6.63
ALS3	Cell wall adhesin	6.61
CAR1	Arginase	6.54
RIM101	Transcription factor	6.27
C3_00020W_A	Unknown function	6.14
GAP6	Broad-specificity amino acid permease	6.06
orf19.3338	Unknown function	6.04

PRA1	Cell surface protein that sequesters zinc from host tissue	5.88
MET3	ATP sulfurlyase	5.83
MRV2	Unknown function	5.83
C4_02930W_A	Unknown function	5.77
CYS3	Cystathionine gamma-lyase	5.77
QDR1	Putative antibiotic resistance transporter	5.71
SAP4	Secreted aspartyl proteinase	5.71
TEC1	TEA/ATTS transcription factor required for biofilm formation	5.64
FAD3	Omega-3 fatty acid desaturase	5.56
SAP5	Biofilm-specific aspartyl protease	5.56
CR_06650C_A	Unknown function	5.42
RTA2	Flippase involved in sphingolipid long chain base release	5.31
PGA25	Putative GPI-anchored adhesin-like protein	5.25
ERG6	Protein involved in ergosterol biosynthesis	5.22
UGA1	Putative transaminase	5.16
PUT1	Putative proline oxidase	4.97
PGA31	Cell wall protein involved in cellular response to drug	4.85
SAP6	Biofilm-specific aspartyl protease	4.81
ALP1	Cystine transporter	4.79
GUT2	Glycerol-3-phosphate dehydrogenase	4.77
C1_07220W_A	Unknown function	4.75
MTM1	Predicted metallochaperone activity	4.59
GNP1	Putative asparagine/glutamine permease;	4.58
RME1	Zinc finger protein; putative meiosis control	4.55
PUT2	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	4.50
AAP1	Putative amino acid permease	4.50
CHA1	Putative catabolic ser/thr dehydratase	4.47
C4_07260W_A	Unknown function	4.47
SUR2	Putative ceramide hydroxylase	4.46
MNN47	Unknown function	4.45
GLY1	L-threonine aldolase	4.44
HAP41	Putative Hap4-like transcription factor	4.36
MNN14	Predicted alpha-1.3-mannosyltransferase activity	4.35
ATO1	Putative transmembrane protein	4.31
YHB5	Flavohemoglobin-related protein	4.31
HEM3	Hydroxymethylbilane synthase	4.28
C2_04080W_A	Unknown function	4.24
C3_03460C_A	Unknown function	4.23
SAP9	Secreted aspartyl protease	4.22
C1_11080W_A	Putative 6-phosphofructo-2-kinase	4.21
MDR1	Plasma membrane multidrug efflux pump	4.14
C4_02770C_A	Predicted ATP. magnesium and ion binding activity	4.12
CAN1	Basic amino acid permease	4.10
C3_00010C_A	Unknown function	4.10
GPR1	Plasma membrane G-protein-coupled receptor	4.09
ECM22	Zn(II)2Cys6 transcription factor	4.06
AOX2	Alternative oxidase	4.02
HWP1	Hyphal cell wall protein	3.97
C6_01780C_A	Predicted chloride transporter	3.97
C6_03670C_A	Unknown function	3.97
HGT12	Glucose. fructose and mannose transporter	3.95
PBR1	Protein required for biological adhesion/biofilm formation	3.92
DED1	Predicted ATP-dependent RNA helicase	3.88
SMM1	Putative dihydrouridine synthase	3.87
ZCF17	Putative Zn(II)2Cys6 transcription factor	3.86
TPK1	cAMP-dependent protein kinase	3.86
EXG2	GPI-anchored cell wall protein	3.79
NCE103	Carbonic anhydrase	3.79

C2_09880C_A	Unknown function	3.76
MAK16	Putative constituent of 66S pre-ribosomal particles	3.73
MSS116	Putative DEAD-box protein	3.73
CDA2	Putative chitin deacetylase	3.73
C6_00290W_A	Unknown function	3.69
TRY6	Helix-loop-helix transcription factor	3.68
CR_09920W_A	Predicted aminoacid transport domain	3.67
orf19.3479	Predicted ORF merged with C6_02350C_A	3.66
GAP5	General amino acid permease	3.65
FAD2	Delta-12 fatty acid desaturase	3.65
C2_06520C_A	Putative thiamine transmembrane transporter	3.64
SEN2	Putative tRNA splicing endonuclease subunit	3.60
NOG2	Putative nucleolar GTPase	3.59
C3_00030C_A	Predicted DEAD-like DNA/RNA helicase	3.54
RPS27A	Ribosomal protein S27	3.54
C1_12470W_A	Unknown function	3.52
C1_02450C_A	Predicted role in cellular bud site selection	3.52
REI1	Putative cytoplasmic pre-60S factor	3.51
CMK1	Putative calcium/calmodulin-dependent protein kinase II	3.50
C2_01760C_A	Unknown function	3.48
PMC1	Vacuolar calcium P-type ATPase	3.47
AGP2	Aminoacid permease	3.46
KTI11	Zn-ribbon protein	3.46
PTR22	Oligopeptide transporter involved in uptake of di-/tripeptides	3.45
MAK21	Putative 66S pre-ribosomal particle subunit	3.44
C3_00040W_A	Unknown function	3.43
C7_02370W_A	Protein with chitin synthesis regulation	3.43
CRD2	Metallothionein	3.42
PHO89	Putative phosphate permease	3.39
CHT2	GPI-linked chitinase	3.38
C2_02580W_A	Predicted MFS membrane transporter	3.36
RPL7	Ribosomal protein L7	3.33
C2_02650C_A	Predicted siroheme synthase middle domains	3.33
C3_04510W_A	Unknown function	3.33
AAH1	Adenine deaminase	3.31
RPC19	Putative RNA polymerases I and III subunit AC19	3.30
HGT10	Glycerol permease involved in glycerol uptake	3.29
SLP3	Putative cation conductance protein	3.26
CR_06350C_A	Unknown function	3.26
CUP9	Transcription factor	3.25
HGT7	Putative MFS glucose transporter	3.24
C1_01130W_A	Putative ubiquitin ligase complex component	3.24
HAC1	bZIP transcription factor	3.22
AGP3	Putativeserinetransporter	3.20
ELF1	Putative mRNA export protein	3.20
GIT2	Putative glycerophosphoinositol permease	3.19
ECM1	Putative pre-ribosomal factor	3.19
RPF2	Putative pre-rRNA processing protein	3.17
UTP13	Putative U3 snoRNA-associated protein	3.15
SOK1	Protein kinase	3.14
C3_03940C_A	Unknown function	3.13
PRO3	Delta 1-pyrroline-5-carboxylate reductase	3.13
PZF1	C2H2 transcriptionfactor	3.13
C3_01290W_A	Unknown function	3.13
C2_05160C_A	Predicted translation repressor activity	3.13
HPT1	Putative hypoxanthine-guanine phosphoribosyltransferase	3.11
C7_02280W_A	Unknown function	3.11
C6_02420W_A	Putative phosphatidyl glycerol phospholipase C	3.10

C5_04910W_A	Protein required for maturation of 18S rRNA	3.08
ABP140	Predicted actin-binding protein	3.08
UTP5	Putative U3 snoRNA-associated protein	3.07
SPB1	Putative AdoMet-dependent methyltransferase	3.06
UME6	Zn(II)2Cys6 transcription factor	3.05
C7_04070C_A	Unknown function	3.04
ATP8	Subunit 8 of the F0 sector of mitochondrial F1F0 ATP synthase	3.03
DBP2	Putative ATP-dependent RNA helicase	3.03
UTP21	Putative U3 snoRNP protein	3.03
WOR4	Predicted C2H2 zinc finger protein	3.02
C2_08910C_A	Protein with a metallo-dependent phosphatase domain	3.02
C2_00130W_A	Unknown function	3.00
C4_06910W_A	PDR-subfamily ABC transporter	3.00
CR_00290W_A	Predicted component of the protein phosphatase type 2A	3.00
C2_06440C_A	Putative peroxisomal membrane proteins	3.00
AUR1	Inositolphosphorylceramide synthase	2.99
MNN4	Regulator of mannosylphosphorylation	2.99
RRP8	Ribosomal protein	2.99
MET10	Sulfite reductase	2.98
CR_04600W_A	Unknown function	2.98
C3_02660W_A	Unknown function	2.97
C1_09330W_A	Putative ribosome-associated protein	2.97
C2_00750W_A	Plasma membrane-associated protein	2.97
DBP3	Putative ATP-dependent RNA helicase	2.97
ZCF1	Zn(II)2Cys6 transcription factor	2.97
C5_01070C_A	Unknown function	2.94
HGC1	Hypha-specific G1 cyclin-related protein	2.94
C7_00490C_A	Putative AdoMet-dependent proline methyltransferase	2.93
C1_11000C_A	Protein with a role in nucleolar integrity	2.92
C6_02100W_A	Unknown function	2.92
C4_06620C_A	Unknown function	2.91
GRF10	Putative homeodomain transcription factor	2.91
NOP15	Nucleolar ribosome biogenesis factor	2.91
MRT4	Putative mRNA turnover protein	2.90
PRN1	Protein similar to pirin; unknown function	2.90
C1_13370W_A	Predicted role in protein catabolic process	2.90
QDR2	Predicted MFS membrane transporter	2.89
C1_00510W_A	Predicted 3-methyl-2-oxobutanoate hydroxymethyltransferase	2.89
C2_02710C_A	Predicted role in rRNA processing	2.88
C1_09040C_A	Putative protein-histidine N-methyltransferase	2.87
C2_02390W_A	Unknown function	2.87
C7_03230C_A	Unknown function	2.86
SOD6	Copper-containing superoxide dismutase	2.85
TAR1	Putative regulator of cellular respiration	2.85
PRN3	Protein similar to pirin; unknown function	2.84
SFL2	Transcription factor required for filamentous growth	2.84
SRP40	Putative chaperone of small nucleolar ribonucleoprotein particles	2.83
DBP7	Putative ATP-dependent RNA helicase	2.83
C3_05160C_A	Predicted role in assembly of precursor of pre-ribosome	2.83
UAP1	UDP-N-acetylglucosamine pyrophosphorylase	2.83
SET6	Unknown function	2.82
C4_00810C_A	Predicted serine-type endopeptidase activity	2.82
PGA37	Putative GPI-anchoredprotein	2.82
CR_00460C_A	Predicted role in pre-18S rRNA processing	2.82
RPF1	Predicted role in the assembly of the large ribosomal subunit	2.81
RLP24	Putative ribosomal protein	2.81
C4_07040W_A	Putative cytochrome P450 protein	2.81
C1_07950C_A	Putative pre-60S pre-ribosomal particle subunit	2.81

UGA3	Zn(II)2Cys6 transcription factor	2.81
C5_02590C_A	Putative mitochondrial membrane protein	2.81
PTC8	Predicted type 2C protein phosphatase (ser/thr-specific)	2.80
SFL1	Transcription factor	2.80
C1_03790C_A	Putative peptidyl-prolyl cis-trans isomerase	2.80
C3_05800W_A	Predicted tRNA-intron endonuclease activity	2.79
C3_06760W_A	Putative role in rRNA processing	2.78
SIK1	Putative U3 snoRNP protein	2.77
TOP1	DNA topoisomerase I	2.77
TES1	Putative acyl-CoA thioesterase	2.76
C7_04090C_A	Predicted mitochondrial cardiolipin-specific phospholipase	2.76
NOP4	Putative nucleolar protein	2.76
NIP7	Putative nucleolar protein	2.76
C1_04040C_A	Putative U3-containing 90S preribosome subunit	2.76
CR_04680C_A	Unknown function	2.76
CSI2	Putative 66S pre-ribosomal particle component	2.76
C3_02040C_A	Predicted nuclear protein	2.75
DPP3	Predicted pyrophosphate phosphatase	2.74
CR_01710W_A	Putative nucleolar protein implicated in ribosome biogenesis	2.74
C3_05450C_A	Unknown function	2.74
ZCF3	Zn(II)2Cys6 domain transcription factor	2.73
NOP6	Putative role in ribosomal small subunit biogenesis	2.73
CNT	CNT family H(+)/nucleoside symporter	2.73
C2_09660W_A	Putative 66S pre-ribosomal particle component	2.73
ENA21	Predicted P-type ATPase sodium pump	2.73
UTP4	Putative U3 snoRNA-associated protein	2.73
C4_02260C_A	Unknown function	2.73
C1_09710C_A	Predicted role in endonucleolytic cleavage	2.72
ERG25	Putative C-4 methyl sterol oxidase	2.72
CHR1	Predicted ATP-dependent RNA helicase	2.71
TBF1	Essential transcription factor	2.71
IDI1	Predicted isopentenyl-diphosphate delta-isomerase activity	2.70
DBP8	Predicted ATP-dependent helicase involved in rRNA processing	2.70
NSA1	Putative 66S pre-ribosomal particles conmponent	2.70
RRP9	Ribosomal protein	2.69
AQY1	Aquaporin water channel	2.69
CYC1	Cytochrome c	2.69
C3_06370C_A	Predicted role in endonucleolytic cleavage	2.68
TAZ1	Putative lyso-phosphatidylcholine acyltransferase	2.68
C6_02290C_A	Putative flavodoxin	2.68
LAG1	Putative ceramide synthase component	2.66
PPT1	Putative serine/threonine phosphatase	2.66
C1_04710C_A	Predicted nucleolar protein	2.65
FRP2	Putativeferricreductase	2.65
C5_04980W_A	Putativeadhesin-likeprotein	2.64
RRP15	Putative nucleolar protein	2.63
NPL3	Putative RNA-binding protein	2.63
WOR3	Transcription factor regulator of phenotypic switching	2.63
C2_04700C_A	U3-containing 90S preribosome complex protein	2.63
FCA1	Cytosine deaminase	2.63
NSA2	Putative protein constituent of 66S pre-ribosomal particles	2.62
C2_09460C_A	Predicted Gal4-like DNA-binding transcription factor	2.62
C1_10950C_A	Putative serine/threonine-protein kinase	2.62
AHR1	Transcription factor involved in regulation of adhesion genes	2.62
GAL7	Putative galactose-1-phoshphate uridyl transferase	2.62
DOG1	Putative 2-deoxyglucose-6-phosphatase	2.62
PTC2	Protein phosphatase of the Type 2C-related family	2.61
NHP2	Putative H/ACA snoRNP protein	2.61

INN1	Predicted protein of the contractile actomyosin ring	2.60
C2_04120C_A	Predicted rRNA binding activity	2.60
NOC4	Putative nucleolar protein	2.59
C3_06680C_A	Unknown function	2.59
UTP20	Putative snoRNA-binding protein	2.59
IPK1	Putative role in organophosphate metabolic process	2.59
C5_03920C_A	Putative rRNA processing protein	2.59
HIS3	Imidazoleglycerol-phosphate dehydratase	2.59
CSA2	Extracellular-associated protein involved in iron assimilation	2.58
CLG1	Putative cyclin-like protein	2.57
C1_14560C_A	Predicted retroviral reverse transcriptase protein	2.57
C1_00160C_A	Putative nucleolar protein	2.57
HMT1	Major type I protein arginine methyltransferase (PRMT)	2.56
ZRT1	Putative zinc transporter	2.56
SUI3	Putative translation initiation factor	2.56
PHO86	Putative endoplasmic reticulum protein	2.55
RHB1	Putative small G protein from the Ras superfamily	2.55
C4_03460C_A	Unknown function	2.55
ECM17	Putative sulfite reductase beta subunit	2.55
C4_07140W_A	Predicted mitochondrial intermembrane space protein	2.54
C5_03970W_A	Alpha/beta-Hydrolase superfamily protein	2.54
HAS1	Predicted ATP-dependent RNA helicase	2.54
C2_06530W_A	Predicted DNA replication licensing factor	2.53
FLC1	Protein involved in heme uptake	2.53
FEN1	Putative fatty acid elongase	2.53
HBR3	Essential protein	2.53
GAR1	Putative H/ACA snoRNP pseudouridylase complex protein	2.53
C1_07960W_A	Predicted nucleic acid binding protein	2.52
HCA4	Putative role in regulation of cell wall biogenesis	2.52
CZF1	Transcription factor	2.52
C3_04260W_A	Predicted role in metal ion transport	2.51
C1_06820W_A	Unknown function	2.51
C4_04520W_A	Predicted enzyme activator and telomerase inhibitor activity	2.51
CR_10470C_A	Predicted rRNA binding activity	2.51
C3_06390W_A	Unknown function	2.51
IMH3	Inosine monophosphate (IMP) dehydrogenase	2.51
CR_04110W_A	Putative RNA-binding protein	2.51
AKR1	Ankyrin-repeat protein	2.50
C3_01430W_A	Unknown function	2.50
TRM2	Putative tRNA methyltransferase	2.49
OPT9	Probable pseudogene similar to OPT1 transporter gene	2.49
C3_02870C_A	Unknown function	2.49
CR_06040W_A	Unknown function	2.49
UTP9	Small-subunit processome protein	2.49
C3_02840W_A	Predicted RNA binding activity	2.49
NMD3	Putative nonsense-mediated mRNA decay protein	2.48
C3_01560W_A	Predicted RNA helicase	2.48
NTH1	Neutral trehalase	2.48
C1_07790C_A	Putative 90S pre-ribosome processome complex subunit	2.48
DOT4	Protein similar to ubiquitin C-terminal hydrolase	2.48
C7_03850W_A	Putative intranuclear transport and DNA replication mediator	2.47
PHO88	Protein with a role in phosphate transport	2.46
PET127	Protein with a predicted role in processing of mitochondrial RNAs	2.46
C3_07980C_A	Unknown function	2.46
RRS1	Putative ribosome biogenesis and nuclear export protein	2.45
DIP2	Putative small ribonucleoprotein complex	2.45
C4_07150W_A	bZIP transcription factor	2.45
NAN1	Putative U3 snoRNP protein	2.45

C7_03590C_A	Putative member of the multi-drug and toxin extrusion family	2.44
C4_05230C_A	Unknown function	2.44
PES1	Putative mRNA-binding protein	2.44
CRZ1	Calcineurin-regulated C2H2 transcription factor	2.44
C1_06540C_A	Exosome non-catalytic core component	2.43
NCS2	Putative cytosolic thiouridylase subunit	2.43
C5_03740W_A	Unknown function	2.43
ARX1	Putative ribosomal large subunit biogenesis protein	2.43
TIM17	Predicted component of the translocase	2.43
FTR2	High-affinity iron permease	2.42
CR_09740W_A	Protein involved in rRNA processing	2.42
MRV4	Unknown function	2.42
C4_06210C_A	Putative GTPase	2.42
DAO2	Putative D-amino acid oxidase	2.42
SAM2	S-adenosylmethionine synthetase	2.42
GDA1	Golgi membrane GDPase	2.42
UTP8	Essential nucleolar protein involved in tRNA export	2.42
C7_01690W_A	Predicted zinc ion binding activity	2.42
C7_00330C_A	Predicted single-stranded telomeric DNA binding activity	2.41
SAS10	Putative processome complex subunit	2.41
IFE2	Putative alcohol dehydrogenase	2.41
C1_10200C_A	Predicted MFS family membrane transporter	2.41
BAS1	Putative Myb-like transcription factor	2.40
C6_02230W_A	Putative GTPase	2.40
NOP1	Nucleolar protein	2.40
C1_03990W_A	Predicted proteasome-mediated protein	2.40
C7_01700W_A	Regulator of calcineurin	2.40
MNN1	Putative alpha-1.3-mannosyltransferase	2.39
PUT4	Putative proline permease	2.39
FCY23	Putative transporter	2.39
ZCF30	Predicted Zn(II)2Cys6 transcription factor	2.39
BMT3	Beta-mannosyltransferase	2.39
CR_00810W_A	Unknown function	2.39
C1_00270W_A	Predicted ORF in retrotransposon Tca8	2.39
SUV3	RNA helicase	2.39
NOP14	Putative nucleolar protein	2.39
C4_00860C_A	Unknown function	2.38
YMX6	Putative NADH dehydrogenase	2.38
UTP18	Putative U3 snoRNA-associated protein	2.38
ALS5	ALS family adhesin	2.38
HXT5	Putative sugar transporter	2.38
SOF1	Putative protein with a predicted role in 40S ribosomal biogenesis	2.37
FYV5	Protein with a predicted role maturation of 18S rRNA	2.37
C2_05750W_A	Predicted rRNA binding activity	2.37
C2_05840W_A	Predicted diphthamide biosynthesis protein	2.37
SGE1	Putative multidrug resistance factor	2.37
C1_04490W_A	Predicted role in nucleosome assembly	2.37
YVH1	Putative dual specificity phosphatase	2.36
SCP1	Putative cortical actin cytoskeleton protein	2.36
CRH11	GPI-anchored cell wall transglycosylase	2.36
GPX2	Predicted glutathione peroxidase	2.36
GFA1	Glucosamine-6-phosphate synthase	2.36
C7_04140C_A	Predicted unfolded protein binding activity	2.36
OAC1	Putative mitochondrial inner membrane transporter	2.36
C1_13210C_A	Unknown function	2.36
RPB8	Putative subunit of RNA polymerases I. II. and III	2.35
MAK5	Putative RNA helicase	2.35
DRE2	Putative cytosolic Fe-S protein assembly protein	2.34

C4_06370C_A	Predicted borate efflux transmembrane transporter activity	2.34
C5_02280C_A	Unknown function	2.34
FAL1	ATP-dependent RNA helicases	2.34
CR_03480W_A	Unknown function	2.34
C4_05330C_A	Putative protein with a predicted role ribosomal biogenesis	2.33
SSF1	Protein involved in ribosome biogenesis	2.33
CRL1	Predicted GTPase	2.33
AGC1	Putative mitochondrial carrier protein	2.33
C4_01500W_A	Protein required for thiolation of uridine	2.33
RAP1	Transcription factor	2.33
C3_03470W_A	Succinate semialdehyde dehydrogenase	2.33
C1_05540C_A	Protein similar to GTPase regulators	2.33
POP3	Putative nuclear RNase component	2.32
C1_11050W_A	Predicted single-stranded nucleic acid binding protein	2.32
C3_05900W_A	Possible G-protein coupled receptor	2.32
C2_06850W_A	Predicted role in pre-rRNA processing	2.32
NOC2	Putative nucleolar complex protein	2.32
C3_04120C_A	Unknown function	2.32
C3_01680C_A	Predicted potassium ion transporter	2.32
C6_01040C_A	ATP-dependent RNA helicase	2.31
GNA1	Glucosamine-6-phosphate acetyltransferase	2.31
C2_08180C_A	Predicted role in ribosomal large subunit biogenesis	2.31
C4_01300W_A	Predicted phosphoprotein phosphatase activity	2.31
C3_02350W_A	Predicted role in endonucleolytic cleavage	2.31
C7_00630C_A	Unknown function	2.30
CR_03360W_A	Predicted role in endonucleolytic cleavage	2.30
C1_06760C_A	Putative U2 snRNP component	2.30
CR_02030C_A	Predicted tRNA (cytosine-5-)-methyltransferase	2.30
MRV1	Unknown function	2.29
C5_01230C_A	Putative aldose 1-epimerase-related protein	2.29
SCH9	Protein kinase	2.29
ENP2	Putative nucleolar protein	2.28
C4_03750C_A	Predicted translation release factor activity	2.28
RPA12	Putative DNA-directed RNA polymerase I	2.28
NDT80	Meiosis-specific transcription factor	2.28
RPS19A	Putative ribosomal protein S19	2.28
C1_04010C_A	Protein with a NADP-dependent oxidoreductase domain	2.28
CR_08940W_A	Predicted tRNA (adenine-N1-)-methyltransferase activity	2.27
RPP1	Predicted subunit of nuclear RNase	2.27
MNN22	Alpha-1.2-mannosyltransferase	2.27
C3_07550C_A	Protein required for mitochondrial ribosome biogenesis	2.27
ADE4	Putative phosphoribosylpyrophosphate amidotransferase	2.27
TRY3	RING-finger transcription factor	2.27
C1_12580W_A	Putative fungal-type vacuole membrane localization	2.26
C4_01220C_A	Protein with a glycoside hydrolase domain	2.26
C6_02350C_A	Putative pseudouridine synthase	2.25
UBI4	Ubiquitin precursor (polyubiquitin)	2.25
C1_12760W_A	Putative nuclear export protein	2.25
CAR2	Ornithine aminotransferase	2.25
CR_00280C_A	Predicted ATPase activity. DNA binding and nucleosome binding activity	2.25
C2_05770W_A	Zn(II)2Cys6 domain transcription factor	2.24
C1_01160C_A	Exosome involved in RNA processing	2.24
C2_06890C_A	Putative oxidoreductase	2.24
NOP8	Predicted role in ribosomal large subunit biogenesis	2.24
ALS6	ALS family protein	2.24
ROD1	Predicted membrane protein with a role in drug tolerance	2.23
SCW4	Putative cel lwall protein	2.23

ELA1	Elongin A	2.23
WOR2	Zn(II)2Cys6 transcription factor	2.23
C3_07430W_A	Predicted acid phosphatase activity	2.22
RLI1	Member of RNase L inhibitor (RLI) subfamily of ABC family	2.22
RPC40	Putative RNA polymerase	2.22
RPS3	Ribosomal protein S3	2.22
C2_01060C_A	Predicted chaperone domain	2.21
CIC1	Putative proteasome-interacting protein	2.21
C7_04080C_A	Putativeadhesin-likeprotein	2.21
NOP5	Predicted involvement in pre-rRNA process	2.21
CR_03320C_A	Unknown function	2.20
C3_04380C_A	Putative U3 snoRNP protein	2.20
C3_01150C_A	Unknown function	2.20
C2_09500W_A	Predicted tRNA dihydrouridine synthase	2.20
C1_13820C_A	Unknown function	2.20
ALS7	ALS family protein	2.20
YHB4	Protein related to flavohemoglobins	2.20
C1_08610C_A	Cytochrome c oxidase subunit	2.20
C4_05010W_A	Protein with a predicted role in ribosome biogenesis	2.19
RPL33	Ribosomal 60S subunitprotein	2.19
PTH2	Putative cAMP-independent regulatory protein	2.19
C5_04940W_A	Maltase	2.19
C3_02180C_A	Deoxyhypusine synthase	2.18
CR_07690W_A	Unknown function	2.18
C4_00250W_A	Unknown function	2.18
SDH4	Succinate dehydrogenase	2.18
C1_10580C_A	Unknown function	2.18
RAC1	G-protein of RAC subfamily	2.18
RAD16	Predicted protein that recognizes and binds damaged DNA	2.18
PAD1	Putative phenylacrylic acid decarboxylase	2.17
C6_03210C_A	RNA polymerase III subunit	2.17
SIM1	Adhesin-like protein	2.17
C3_03130C_A	Predicted role in cell wall biogenesis	2.17
RSM22	Predicted mitochondrial small ribosomal subunit	2.17
C1_10190W_A	Predicted nucleotide binding activity	2.17
C3_02630C_A	Unknown function	2.17
DOT6	Protein with a predicted role in telomeric gene silencing	2.16
RRN11	Putative RNA polymerase I subunit	2.16
TRM9	Putative tRNA methyltransferase	2.16
SUT1	Zn2Cys6 transcription factor involved in sterol uptake	2.16
C7_01510W_A	Predicted membrane transporter	2.16
WOR1	Transcription factor of white-opaque phenotypic switching	2.16
HAP2	Transcription factor that regulates low-iron induction	2.16
SRD1	Sur7 family protein with Pall-like domains	2.16
KRE30	YEF3-subfamily ABC family protein	2.15
SUI1	Putative translation initiation factor	2.15
UPC2	Zn2-Cys6 transcript factor;	2.15
C6_01890C_A	Putative helicase	2.15
PEL1	Predicted enzyme of mitochondrial phospholipid biosynthesis	2.15
C1_12570C_A	Putative elongator complex subunit	2.15
C4_06850C_A	Predicted mRNA binding and metalloaminopeptidase activity	2.15
HNM1	Putative choline/ethanolamine transporter	2.15
C1_06480C_A	Predicted role in ER to Golgi vesicle-mediated transport	2.15
SEC9	t-SNARE protein required for secretory vesicle-membrane fusion	2.14
NOG1	Putative GTPase	2.14
CR_03980W_A	Unknown function	2.14
CR_03310C_A	Putative transcription factor with zinc finger DNA-binding motif	2.14
PWP2	Putative 90S pre-ribosomal component	2.14

GIT1	Glycerophosphodiestertransporter	2.14
GPD2	Surface protein similar to glycerol 3-P dehydrogenase	2.14
C3_04730C_A	Unknown function	2.14
YMC1	Putative inner mitochondrial membrane transporter	2.14
C5_04840C_A	Putative ribosome biogenesis factor	2.14
MRS4	Mitochondrial carrier family member	2.13
MRV6	Unknown function	2.13
C2_00050C_A	Unknown function	2.13
C1_13090W_A	Unknown function	2.12
CR_07080W_A	Predicted constituent of 66S pre-ribosomal particles	2.12
C1_09840C_A	Protein involved in ubiquitin-mediated protein degradation	2.12
CR_01160W_A	Unknown function	2.12
LAC1	Ceramide synthase	2.12
C5_04850W_A	Unknown function	2.12
RPA34	Putative RNA polymerase I subunit	2.12
SEC14	Essential protein	2.12
C2_01730W_A	Putative involvement in chromosome segregation	2.11
SWE1	Putative protein kinase required for virulence	2.11
NAR1	Putative cytosolic iron-sulfur protein assembly machinery protein	2.11
C3_02390W_A	Unknown function	2.11
CR_05450C_A	Unknown function	2.11
GNP3	Putative high-affinity glutamine permease	2.10
CR_02440W_A	Unknown function	2.10
C4_03740W_A	Predicted role in U4 snRNA 3'-end processing	2.10
DIM1	Putative 18S rRNA dimethylase	2.10
C1_10050W_A	Unknown function	2.10
C2_01370C_A	Unknown function	2.10
C7_01910C_A	Unknown function	2.09
CR_10170C_A	Putative asparaginase	2.09
NRG2	Transcription factor	2.09
PDE1	Low affinity cyclic nucleotide phosphodiesterase	2.09
C1_11280W_A	Predicted pre-mRNA binding activity	2.09
C5_02510C_A	Predicted nucleotide binding activity	2.09
PDE2	High affinity cyclic nucleotide phosphodiesterase	2.09
CHS2	Chitin synthase	2.09
ISA1	Putative mitochondrial iron-sulfur protein	2.08
C4_00530C_A	Predicted hydrolase activity	2.08
BMS1	Putative GTPase	2.08
C1_07660W_A	Unknown function	2.08
JIP5	Predicted role in biogenesis of the large ribosomal subunit	2.08
CR_01950W_A	Predicted RNA polymerase I activity	2.08
RRP6	Putative nuclear exosome exonuclease component	2.08
C2_10020C_A	Predicted catalytic activity	2.08
CR_07170W_A	Putative dehydrogenase	2.08
C1_06800W_A	Predicted RNA pyrophosphohydrolase	2.08
C5_04500W_A	Unknown function	2.07
ZFU2	Zn(II)2Cys6 transcription factor	2.07
TPO3	Putative polyamine transporter	2.07
DRS1	Putative nucleolar protein	2.07
ADE2	Phosphoribosylaminoimadazole carboxylase	2.07
C2_05960C_A	Unknown function	2.07
C1_05220C_A	Predicted nucleotide binding activity	2.07
UTP15	Small subunit (SSU) processome component	2.06
C7_03210W_A	Unknown function	2.06
KTR2	Mannosyltransferase	2.06
C4_02380W_A	Unknown function	2.06
C2_05520W_A	Predicted protein-lysine N-methyltransferase activity	2.06
CR_04170W_A	Predicted rRNA (cytosine-C5-)-methyltransferase activity	2.06

MTG1	Putative mitochondrial GTPase	2.06
YTM1	Predicted involvement in biogenesis of large ribosomal subunit	2.06
C2_09510C_A	Predicted ATP:ADP antiporter activity	2.06
C2_00940W_A	Plasma membrane-associated protein	2.05
PHO100	Putative inducible acid phosphatase	2.05
PGA10	GPI anchored membrane protein	2.05
C2_00280C_A	Putative pre-mRNA-splicing factor	2.05
C2_05040C_A	Unknown function	2.05
C1_07530W_A	Unknown function	2.05
ZCF2	Zn(II)2Cys6 transcription factor	2.04
C1_04510W_A	Putative transcription factor with bZIP DNA-binding motif	2.04
C4_02720C_A	Putative plasma membrane protein	2.04
C5_00510W_A	Unknownfunction	2.04
CIS2	Putative role in regulation of biogenesis of the cell wall	2.04
CR_01510C_A	Unknown function	2.04
C7_01030C_A	Predicted mRNA binding activity	2.04
POP4	Putative nuclear RNase component	2.04
CR_00790C_A	Unknown function	2.04
C5_00790C_A	Putative RNA-binding protein	2.03
C5_02440C_A	Predicted protein serine/threonine kinase activity	2.03
ELP3	Predicted histone acetyltransferase	2.03
IFD6	Aldo-keto reductase	2.03
MXR1	Putative methionine sulfoxide reductase	2.03
C3_05140C_A	Predicted tRNA (guanine-N2-)-methyltransferase activity	2.03
C1_10970W_A	Putative nucleolar protein	2.03
PGA11	Putative GPI-anchored protein; unknown function	2.03
C7_02630W_A	Unknown function	2.03
RPC53	Predicted RNA polymerase III activity	2.03
C3_05750C_A	Unknown function	2.03
C1_05950C_A	Unknown function	2.03
NMA111	Putative serine protease and general molecular chaperone	2.02
YOR1	Predicted ABC-type plasma membrane transporter	2.02
ENP1	Protein required for pre-rRNA processing	2.02
C1_10620W_A	Predicted pseudouridine synthase	2.02
C2_02300W_A	Proten with a predicted role in mRNA splicing	2.02
C1_08710W_A	Unknown function	2.01
C5_02020C_A	Predicted role in RNA polymerase II complex import to nucleus	2.01
CYC3	Mitochondrial cytochrome c heme lyase	2.01
C3_07420W_A	Predicted eukaryotic 43S preinitiation complex	2.01
BRG1	Transcription factor	2.01
CFL2	Putative oxiredutase	2.01
C6_01600W_A	Unknown function	2.01
C2_02540W_A	Predicted RNA binding protein involved in synthesis of rRNAs	2.01
TSR1	Component of 20S pre-rRNA processing unit	2.01
SDA1	Predicted nuclear protein involved in actin cytoskeleton	2.01
ERG251	C-4 sterol methyl oxidase	2.01
C1_12680W_A	Predicted unfolded protein binding activity	2.01
RNR3	Putative ribonucleotide reductase large subunit	2.00
C2_04570W_A	Predicted mRNA binding activity	2.00
MSU1	Predicted exoribonuclease II activity	2.00
C2_00410C_A	Predicted ATP-dependent RNA helicase	2.00
RMP1	Predicted RNase subunit that processes pre-rRNA	2.00

Down-regulated genes

Gene	Biological function	$\frac{\text{mRNA}_{\text{biofilm}}}{\text{mRNA}_{\text{planktonic}}}$
DUR3	High affinity spermidine transporter	0.05

MEP2	Ammonium permease	0.09
JEN2	Dicarboxylic acid transporter	0.11
C2_00180C_A	Predicted uricase	0.15
C1_01630W_A	Putative mitochondrial protein	0.15
FDH1	Formate dehydrogenase	0.17
C1_07160C_A	Protein conserved among the CTG-clade	0.17
CWH8	Putative dolichyl pyrophosphate (Dol-P-P) phosphatase	0.18
HSP30	Putative heat shock protein	0.18
LAP3	Putative aminopeptidase	0.18
CTN1	Carnitine acetyl transferase	0.18
C2_01660C_A	Unknown function	0.18
CCP1	Putative Cytochrome-c peroxidase	0.18
MDH1-1	Predicted malate dehydrogenase precursor	0.18
C5_03770C_A	Putative formate dehydrogenase	0.18
C5_03710C_A	Unknown function	0.18
DUR1,2	Urea amidolyase involved in response to abiotic stimulus	0.19
ADH2	Alcohol dehydrogenase;	0.19
PGA13	GPI-anchored cell wall protein involved in cell wall synthesis	0.19
C2_01540W_A	Predicted sulfonate dioxygenase	0.20
UCF1	Predicted involvement in generation of energy	0.21
SOD4	Cu-containing superoxide dismutase	0.22
GRE3	Putative D-xylose reductase	0.22
HPD1	3-hydroxypropionate dehydrogenase	0.23
FET31	Putative multicopper oxidase	0.23
MMD1	Mitochondrial protein required for transamination of isoleucine	0.23
FMP45	Predicted membrane protein induced during mating	0.24
DAK2	Putative dihydroxyacetone kinase	0.24
HGT19	Putative MFS glucose/myo-inositol transporter	0.24
C1_10280C_A	Unknown function	0.24
AMO1	Putative peroxisomal copper amine oxidase	0.25
C1_03510C_A	Unknown function	0.25
C5_00100C_A	Unknown function	0.25
ADH3	Putative NAD-dependent (R.R)-butanediol dehydrogenase	0.26
C5_02110W_A	Putative heat shock protein	0.26
OSM2	Putative mitochondrial fumarate reductase	0.26
C1_10360C_A	Unknown function	0.27
PNG2	Putative peptide:N-glycanase	0.27
RSD1	Predicted short chain dehydrogenase	0.27
C2_09820W_A	Unknown function	0.27
C2_00860C_A	Unknown function	0.27
SOD2	Mitochondrial Mn-containing superoxide dismutase	0.27
DLD1	Putative D-lactate dehydrogenase	0.27
AGO1	Putative Argonaute protein involved in RNA silencing	0.28
NGT1	N-acetylglucosamine (GlcNAc)-specific transporter	0.28
C1_14090W_A	Putative role in chaperone-mediated protein assembly	0.28
C6_02030C_A	Unknown function	0.28
C3_03760W_A	Putative diacylglycerol acyltransferase	0.28
PDC11	Pyruvate decarboxylase	0.28
ZSF1	Predicted mRNA-binding protein	0.29
GLT1	Putative glutamate synthase	0.29
CR_09140C_A	Protein with a role in directing meiotic recombination events	0.29
PRC2	Putative carboxypeptidase	0.29
C2_07070W_A	Putative quinone oxidoreductase	0.29
C3_07470W_A	Putative plasma membrane protein with role in cell wall integrity	0.29
C5_00330C_A	Predicted role in fermentation and protein maturation	0.29
C1_01930W_A	Unknown function	0.30
AHP1	Alkyl hydroperoxide reductase	0.30
PSA2	Mannose-1-phosphate guanyltransferase	0.30

MSH2	Putative DNA mismatch repair factor	0.30
CR_08920W_A	Predicted oxidoreductase and dehydrogenase domains	0.30
C1_12180C_A	Unknown function	0.30
IHD2	Unknown function	0.31
PEP1	Type I transmembrane	0.31
C1_11920W_A	Predicted ATPase activator activity	0.31
C1_10310W_A	Predicted methyltransferase	0.31
PXP2	Putative acyl-CoA oxidase	0.31
ARO10	Aromatic decarboxylase	0.31
HGT18	Putative glucose transporter of the major facilitator superfamily	0.31
GND1	6-phosphogluconate dehydrogenase	0.31
MCR1	NADH-cytochrome-b5 reductase	0.31
C1_01620C_A	Predicted oxidoreductase activity and role in metabolic process	0.32
RVS162	Protein containing a BAR domain	0.32
C4_01840C_A	Putative dienelactone hydrolase	0.32
C2_07630C_A	Putative stress protein	0.32
C1_13190W_A	Putative mitochondrial membrane protein of unknown function	0.32
ROT2	Alpha-glucosidase II	0.32
TFS1	Putative carboxypeptidase	0.32
C1_00410C_A	Hexadecenal dehydrogenase	0.32
ATP2	F1 beta subunit of F1F0 ATPase complex	0.33
C6_03320W_A	Unknown function	0.33
IDP2	Isocitrate dehydrogenase	0.33
TRY4	C2H2 transcription factor	0.33
C7_00590W_A	Unknown function	0.33
PRX1	Thioredoxin peroxidase	0.33
C7_03560W_A	Unknown function	0.33
HEM13	Coproporphyrinogen III oxidase	0.33
C3_07490W_A	Predicted phosphopentomutase activity	0.33
FUM11	Fumarate hydratase	0.34
ENO1	Enolase involved in glycolysis and gluconeogenesis	0.34
ADE17	5-Aminoimidazole-4-carboxamide ribotide transformylase	0.34
C1_02780W_A	Predicted dienelactone hydrolase domain	0.34
C2_05570C_A	Putative lipid raft associated protein	0.34
C3_06040W_A	CCCH zinc finger protein	0.34
C4_03050C_A	Predicted carboxypeptidase activity	0.34
ECM15	Unknown function	0.34
ALD6	Putative aldehyde dehydrogenase	0.34
C2_09590C_A	Predicted copper uptake transmembrane transporter activity	0.35
ATP1	ATP synthase alpha subunit	0.35
GCN2	Translation initiation factor 2-alpha (eIF2alpha) kinase	0.35
CCN1	G1 cyclin	0.35
MLS1	Malate synthase	0.35
HAK1	Putative potassium transporter	0.35
HSP31	Putative heat shock protein	0.35
FET99	Multicopper oxidase family protein	0.35
CYB2	Putative cytochrome b2 precursor	0.35
CR_09010C_A	Putative cystathionine gamma-synthase	0.35
RIB5	Putative riboflavin synthase	0.36
FAV2	Adhesin-like protein	0.36
SOD3	Cytosolic manganese-containing superoxide dismutase	0.36
C4_00420C_A	Predicted enzyme inhibitor activity	0.36
CR_01080W_A	Unknown function	0.36
ZRT3	Unknown function	0.36
CR_09070C_A	Unknown function	0.36
TDH3	NAD-linked glyceraldehyde-3-phosphate dehydrogenase	0.36
GAD1	Putative glutamate decarboxylase	0.36
C6_02660C_A	Predicted amidase activity	0.36

C1_08340C_A	Sorting nexin	0.36
PGA38	Putative adhesin-like GPI-anchored protein	0.36
HGT17	Putative MFS family glucose transporter	0.36
C3_03680W_A	Predicted sphinganine-1-phosphate aldolase activity	0.36
C1_09980C_A	Predicted acylglycerol lipase activity	0.37
SCW11	Cell wall protein. possibly an essential gene	0.37
C2_02790C_A	Predicted amino acid transmembrane transporter	0.37
C2_09650W_A	Predicted poly(A) binding activity	0.37
MCD1	Alpha-kleisin cohesin complex subunit	0.37
GLK4	Putative glucokinase	0.37
CCS1	Copper chaperone	0.37
GVP36	BAR domain protein	0.37
GTT11	Glutathione S-transferase	0.37
COF1	Putative cofilin	0.37
PNP1	Purine nucleoside phosphorylase	0.37
CR_03530W_A	Mitochondrial inner membrane protein of unknown function	0.38
XYL2	D-xylulose reductase	0.38
HSP60	Heat shock protein	0.38
CIT1	Citrate synthase	0.38
ZRT2	Zinc transporter	0.38
PGA4	GPI-anchored cell surface protein	0.38
HOM6	Putative homoserine dehydrogenase	0.38
CR_08850W_A	Unknown function	0.38
SER1	Putative 3-phosphoserine aminotransferase	0.38
MDH1	Mitochondrial malate dehydrogenase	0.38
LYS12	Homoisocitrate dehydrogenase	0.38
CR_09590W_A	Predicted role in ubiquinone-6 biosynthetic process	0.38
C5_00080C_A	Epsilon-COP subunit of the coatomer	0.38
HSM3	Predicted role in mismatch repair	0.38
C3_02340W_A	Unknown function	0.38
KAR9	Predicted role in mitotic spindle positioning	0.39
CWH43	Putative sensor/transporter protein with a predicted role in cell wall biogenesis	0.39
GLK1	Putative glucokinase	0.39
HSP70	Putative hsp70 chaperone	0.39
TKL1	Putative transketolase	0.39
CTA9	Protein required for normal filamentous growth	0.39
GAT1	GATA-type transcription factor	0.39
C1_03100W_A	Putative lipid-binding protein	0.39
GLO1	Putative monomeric glyoxalase I	0.39
C3_03570C_A	Predicted oxidoreductase	0.39
SAM51	Component involved in mitochondrial protein import	0.39
AHP2	Putative thiol-specific peroxiredoxin	0.40
SEC61	ER protein-translocation complex subunit	0.40
FBA1	Fructose-bisphosphate aldolase	0.40
C2_01450C_A	Unknown function	0.40
NCR1	Putative vacuolar membrane protein	0.40
MSH6	Predicted involvement in mismatch repair	0.40
NIT3	Putative nitrilase	0.40
C1_10680C_A	Predicted lipid-binding ER protein	0.40
C6_02140W_A	Unknown function	0.40
C5_03800W_A	Cytochrome c oxidase subunit	0.40
C6_01360W_A	Unknown function	0.40
C1_11990W_A	Putative cell wall adhesin-like protein	0.40
C1_12720C_A	Unknown function	0.40
CR_06510W_A	Unknown function	0.40
C1_09670C_A	Predicted DNA binding activity	0.40
CTR1	Copper transporter	0.40

C2_02620W_A	Putative mitochondrial protein of unknown function	0.40
IST2	Predicted lipid binding activity	0.40
C7_03150W_A	Unknown function	0.40
CAS4	cell wall integrity signaling network protein	0.41
C3_05290C_A	Unknown function	0.41
SEC23	Putative GTPase-activating protein	0.41
RBE1	Pry family cell wall protein	0.41
C3_05850W_A	Predicted ubiquitin-protein transferase activity	0.41
MEC1	Cell cycle checkpoint protein with a role in genome integrity	0.41
CHC1	Clathrin heavy chain	0.41
RIC1	Predicted guanyl-nucleotide exchange factor	0.41
PCT1	Putative choline-phosphate cytidylyl transferase	0.41
CR_07350W_A	Predicted role in ER-dependent peroxisome organization	0.41
RGS2	Protein of RGS superfamily	0.41
GCY1	Aldo/keto reductase	0.41
DQD1	Putative 3-dehydroquinate dehydratase	0.42
ETR1	Putative 2-enoyl thioester reductase	0.42
CDC19	Pyruvate kinase at yeast cell surface	0.42
C6_03880W_A	Predicted oxidoreductase activity	0.42
C2_04480W_A	Predicted oxidoreductase activity	0.42
GPH1	Putative glycogen phosphorylase	0.42
MVD	Mevalonate diphosphate decarboxylase	0.42
VMA10	Subunit of the peripheral membrane domain of H(+)-ATPase	0.42
C1_07080W_A	Suggested role in chromosome maintenance	0.42
TPS3	Predicted trehalose-phosphate synthase	0.42
CAS1	Putative transcription factor	0.42
SRV2	Adenylate cyclase-associated protein	0.42
SSD1	Protein with role in resistance to host antimicrobial peptides	0.42
OBPA	Putative oxysterol binding protein	0.42
CR_09700W_A	Predicted membrane transporter of major facilitator superfamily	0.42
KRE5	UDP-glucose:glycoprotein glucosyltransferase	0.42
PRC3	Putative carboxypeptidase Y precursor	0.42
CR_03470W_A	Unknown function	0.42
C1_04200C_A	Predicted structural constituent of nuclear pore activity	0.42
C4_03370C_A	Unknown function	0.42
TPO4	Putative sperimidine transporter	0.42
C7_00430W_A	Putative ferric reductase	0.43
C3_01130C_A	ZZ-type zinc finger protein	0.43
GPM1	Phosphoglycerate mutase	0.43
ATP4	Putative F0-ATP synthase subunit 4	0.43
POL30	Proliferating cell nuclear antigen (PCNA)	0.43
TAL1	Transaldolase	0.43
VMA13	Predicted proton-transporting ATPase	0.43
C1_06860W_A	Predicted role in response to stress	0.43
YKE2	Possible heterohexameric Gim/prefoldin protein	0.43
PGI1	Predicted Glucose-6-phosphate isomerase	0.43
C7_02920W_A	Unknown function	0.43
PGA63	Component COPII vesicle coat	0.43
C1_09310C_A	Unknown function	0.43
CRM1	Predicted involvement in protein nuclear export	0.43
LEU2	Isopropyl malate dehydrogenase	0.43
PMT4	Protein mannosyltransferase	0.44
COQ6	Predicted oxidoreductase activity	0.44
DPM1	Dolichol-phosphate mannose synthase catalytic subunit	0.44
CR_06850C_A	Unknown function	0.44
C4_01830C_A	Predicted metallocarboxypeptidase	0.44
CDC10	Septin	0.44
NUP82	Linker nucleoporin of the nuclear pore complex	0.44

C4_02340W_A	Putative protease B inhibitor	0.44
C6_02490C_A	Unknown function	0.44
C1_02770W_A	Unknown function	0.44
EMP24	COPII-coated vesicle component	0.44
SLA2	Actin binding protein	0.44
C2_00770W_A	Unknown function	0.44
CR_00820C_A	Putative ribosome binding activity	0.44
TPK2	cAMP-dependent protein kinase catalytic subunit	0.44
C7_00870W_A	Putative guanine deaminase	0.44
C1_02730W_A	Unknown function	0.44
C3_06640W_A	Unknown function	0.44
MRF1	Putative mitochondrial respiratory protein	0.44
CLN3	G1 cyclin	0.44
FBP1	Fructose-1.6-bisphosphatase	0.44
C1_11860W_A	Predicted essential RNA-binding G protein	0.44
C6_03260W_A	Putative ribonuclease H1	0.44
C2_04400W_A	Unknown function	0.44
TRA1	Subunit of the NuA4 histone acetyltransferase complex	0.45
TIP120	Putative involvement in regulation of SCF complexes	0.45
NAG1	Glucosamine-6-phosphate deaminase	0.45
C6_04420W_A	Unknown function	0.45
C4_04020C_A	Unknown function	0.45
CTA26	Putative transcription factor/activator	0.45
SEC5	Predicted exocyst component	0.45
USO1	Predicted role in ER to Golgi vesicle-mediated transport	0.45
COX6	Putative cytochrome c oxidase	0.45
TEL1	Predicted se/thr kinase and telomeric DNA binding activity	0.45
C5_02560C_A	Predicted ATP binding and protein kinase activity	0.45
CR_02800C_A	Unknown function	0.45
SOD1	Cytosolic copper- and zinc-containing superoxide dismutase	0.45
LPG20	Aldo-keto reductase family protein	0.45
CRZ2	C2H2 transcription factor	0.45
HSP21	Heat shock protein	0.45
CFL5	Predicted Ferricreductase	0.45
GWT1	Inositol acyltransferase	0.45
C2_03260W_A	Unknown function	0.45
VMA2	Vacuolar H(+)-ATPase	0.45
CDR4	Putative ABC transporter superfamily	0.45
C1_12880C_A	Predicted DNA and nucleic acid binding activity	0.45
C1_10560C_A	Unknown function	0.45
CLB4	B-type mitotic cyclin	0.45
C1_04430C_A	Unknown function	0.45
CR_03120W_A	Mitochondrial intermembrane space protein of unknown function	0.45
BNI1	Formin	0.45
SKI2	Predicted mRNA binding activity	0.45
RTG1	RNA polymerase II transcription factor	0.46
VPS35	Putative role in vacuolar sorting	0.46
ACH1	Acetyl-coA hydrolase	0.46
PMA1	Plasma membrane H(+)-ATPase	0.46
C5_02140C_A	Predicted pyridoxal 5'-phosphate synthase	0.46
C6_00910C_A	Protein with a predicted thioredoxin-like domain	0.46
C2_05550W_A	Protein with a predicted cytochrome binding domain	0.46
CR_05500C_A	Unknown function	0.46
C4_04290W_A	Exocyst subunit involved in polarized growth	0.46
DCK2	Predicted role in filamentous growth	0.46
CR_03690W_A	Predicted hydrolase activity	0.46
VMA7	Putative subunit of the V-ATPase complex	0.46
CR_00220W_A	Predicted electron carrier activity	0.46

C7_00340C_A	Putative transporter activity	0.46
C2_07270W_A	Protein with an FMN-binding domain	0.46
HGT3	Putative glucose transporter of the major facilitator superfamily	0.46
CR_10020C_A	Predicted oligosaccharide binding activity	0.46
HSP90	Essential chaperone	0.46
C4_03500C_A	Unknown function	0.46
TPS1	Trehalose-6-phosphate synthase	0.46
CR_05200C_A	Unknown function	0.46
CTN3	Peroxisomal carnitine acetyl transferase	0.46
C6_04100W_A	Predicted phosphatidylinositol binding activity	0.46
SOL3	Putative 6-phosphogluconolactonase	0.46
BUL4	Unknown function	0.47
NSP1	Essential component of the nuclear pore complex	0.47
C1_06250W_A	Unknown function	0.47
DIT2	Monooxygenase of the cytochrome P450 family	0.47
STI1	Predicted involvement in Cdc37 chaperone activity	0.47
IPP1	Putative inorganic pyrophosphatase	0.47
NIP100	p150 subunit of dynactin	0.47
BRO1	Class E vacuolar protein sorting factor	0.47
C1_12170C_A	Unknown function	0.47
C4_00910C_A	Putative mutator-like element (MULE) transposase	0.47
C1_04560W_A	Putative transcription factor with DNA-binding motif	0.47
SEC22	Predicted role in ER to Golgi vesicle-mediated transport	0.47
QCR2	Ubiquinol-cytochrome-c reductase	0.47
C3_06810W_A	Predicted ubiquitin-protein transferase activity	0.47
C1_11120C_A	Unknown function	0.47
COX9	Putative subunit VIIa of cytochrome c oxidase	0.47
VPS16	Putative involvement in protein-vacuolar targeting	0.47
SRO77	Predicted role in fusion of Golgi vesicles to the plasma membrane	0.47
C1_04780C_A	Predicted role in negative regulation of TORC1 signaling	0.47
C5_04320C_A	RNI-like superfamily domain-containing protein	0.47
C7_03370C_A	Unknown function	0.47
MAM33	Putative mitochondrial acidic matrix protein	0.47
C7_04280C_A	Putative catechol o-methyltransferase	0.47
C3_07740W_A	Predicted COPI-coated vesicle	0.47
RIM20	Protein involved in the pH response pathway	0.47
HAT2	Putative Hat1-Hat2 histone acetyltransferase complex subunit	0.47
BMH1	Unknown function	0.47
C7_01650W_A	RNA polymerase III transcription initiation factor complex subunit	0.47
C1_02220C_A	Unknown function	0.47
C5_03950W_A	Putative Rab GTPase activator	0.47
SAR1	Predicted involvement in ER-to-Golgi protein transport	0.47
C5_02050W_A	Predicted role in Golgi to endosome transport	0.47
CR_05320C_A	Unknown function	0.47
ARC18	Putative ARP2/3 complex subunit	0.47
BPH1	Unknown function	0.47
CHS5	Putative chitin biosynthesis protein	0.47
C5_04050W_A	Plasma membrane-localized protein of unknown function	0.47
PNC1	Putative nicotinamidase	0.47
ATP14	Putative mitochondrial F1F0 ATP synthase subunit	0.48
C2_04500W_A	Predicted ATPase activity and DNA binding activity	0.48
LHS1	Predicted Kex2p substrate	0.48
LYS9	Saccharopine dehydrogenase	0.48
C3_01720C_A	Alpha subunit of COPI vesicle coatomer complex	0.48
C6_04250W_A	Predicted cytochrome-c oxidase activity	0.48
C5_05350W_A	Predicted DNA primase activity	0.48
C2_00760C_A	Unknown function	0.48
MED16	Putative RNA polymerase II mediator complex subunit	0.48

SPS20	Peroxisomal 2.4-dienoyl-CoA reductase	0.48
C2_01690W_A	Putative succinate dehydrogenase	0.48
C1_09060C_A	Unknown function	0.48
C4_04800W_A	Predicted role in ER-dependent peroxisome organization	0.48
ATP3	F1-ATP synthase complex subunit	0.48
RNR21	Ribonucleoside-diphosphate reductase	0.48
C6_02190C_A	Putative serine/threonine/tyrosine (dual-specificity) kinase	0.48
SEC24	Protein with a possible role in ER to Golgi transport	0.48
PDK2	Putative pyruvate dehydrogenase kinase	0.48
C3_03410C_A	Putative glycoside hydrolase	0.48
C7_03030W_A	Unknown function	0.48
C3_03590W_A	Predicted role in inner mitochondrial membrane organization	0.48
BZZ1	Protein involved in the regulation of actin polymerization	0.48
ZWF1	Glucose-6-phosphate dehydrogenase	0.48
LYS22	Homocitrate synthase	0.49
PHB1	Putative prohibitin	0.49
HNT1	Putative protein kinase C inhibitor-I	0.49
C3_04420W_A	Predicted ubiquitin-protein ligase	0.49
GCN4	bZIP transcription factor	0.49
TOM1	Putative E3 ubiquitin ligase	0.49
RAD50	Putative DNA double-strand break repair factor	0.49
CDC3	Septin	0.49
C4_05590W_A	Unknown function	0.49
ARP3	Protein with Myo5p-dependent localization at hyphal tip	0.49
SMC1	Putatve chromosomal ATPase	0.49
C1_09520C_A	Unknown function	0.49
TLO4	Telomere-proximal gene of unknown function	0.49
MDH1-3	Predicted malate dehydrogenase	0.49
CR_05860W_A	Unknown function	0.49
C6_03050C_A	Unknown function	0.49
HSL1	Putative protein kinase	0.49
TPD3	Subunit of protein serine/threonine phosphatase PPA2	0.49
GDH3	NADP-glutamate dehydrogenase	0.49
C4_01510W_A	Predicted carbonyl reductase (NADPH) activity	0.49
IFD3	Putative aldo/keto reductase	0.49
C7_00790W_A	Predicted phosphatidylinositol-3-phosphate binding activity	0.49
SMI1B	Putative cell wall assembly regulatory protein	0.49
CR_08560C_A	Predicted SUMO transferase activity	0.49
MCM3	Putative DNA replication protein	0.49
C5_02330W_A	Unknown function	0.50
GLR1	Glutathione reductase	0.50
FUM12	Putative fumarate hydratase	0.50
C3_02750W_A	Protein with a ribonuclease III domain	0.50
C7_01710W_A	Membrane-localized protein of unknown function	0.50
MON2	Peripheral membrane protein	0.50
WBP1	Putative oligosaccharyltransferase subunit	0.50
C1_04860W_A	Unknown function	0.50
C5_05020C_A	Dolichyl-diphosphooligosaccharide-protein glycotransferase	0.50
LSC1	Putative succinate-CoA ligase subunit	0.50

Table AIII.3 Regulatory associations between biofilm-induced genes and biofilm-related transcriptions factors. The genes found to be up-regulated in *C. albicans* SC5314 biofilms developed 24 h in RPMI at pH 4, in comparison with planktonic cells, were clustered according to the regulatory associations with transcription factors mediating the control of transcriptome-wide alterations related with biofilm formation, based on the information available on the PathoYeastract database. Genes found to be up-regulated specifically at the acidic conditions used in this study are highlighted in grey.

Bcr1 (98 targets genes)				
ENA2	*PTR22*	*PTH2*	*ECM22*	*GPD2*
C1_01510W_A	*ADE2*	*CHT2*	*SCW4*	*SAP6*
HGT1	*TEC1*	*SFL2*	*IFD6*	*ALS5*
HGT2	*C3_04730C_A*	*C5_04850W_A*	*AOX2*	*ALS2*
DIP5	*C3_05160C_A*	*C5_04940W_A*	*HYR1*	*C7_01510W_A*
BRG1	*WOR2*	*GRF10*	*SIM1*	*ENA21*
UME6	*RHB1*	*C6_00290W_A*	*SOD6*	*AQY1*
RME1	*C3_05450C_A*	*GPX2*	*SOD5*	*IFE2*
SWE1	*AHR1*	*TOP1*	*SAP9*	*ALS3*
WOR1	*ALS6*	*CAN1*	*PGA10*	*GPN1*
C1_10580C_A	*C3_07550C_A*	*SAP5*	*PHO89*	
FCY23	*WOR4*	*SAP4*	*CRH11*	
RIM101	*C4_02720C_A*	*ALS1*	*ECE1*	
HGT7	*PGA31*	*HGT10*	*HWP1*	
C2_01760C_A	*HAP41*	*IHD1*	*PHR1*	
SIT1	*MNN4*	*C7_00630C_A*	*CFL2*	
C2_09460C_A	*GUT2*	*CUP9*	*CSA2*	
NCE103	*CZF1*	*WOR3*	*ZRT1*	
ZCF1	*C5_00510W_A*	*CR_04680C_A*	*PRA1*	
C3_02660W_A	*CYC3*	*RNR3*	*PGA37*	
C3_03460C_A	*GPR1*	*SFL1*	*PGA25*	
C3_03470W_A	*HIP1*	*SOK1*	*C1_08610C_A*	

Ume6 (52 target genes)		
HGC1	*SAP6*	*AQY1*
EXG2	*DPP3*	*YVH1*
UME6	*CR_06040W_A*	*NOC2*
HYR1	*CHS2*	*ALS3*
MET3	*C1_00270W_A*	*UTP5*
SOD5	*SIM1*	*C1_05540C_A*
C2_05750W_A	*C2_04080W_A*	*C1_09710C_A*
MAK16	*C3_01560W_A*	*C2_10020C_A*
C2_06530W_A	*CAR2*	
CYC1	*HWP1*	
GFA1	*PHR1*	
C3_04120C_A	*GAP5*	
ERG251	*NOP1*	
IDI1	*MNN1*	
CRH11	*C5_04980W_A*	
ECE1	*SDH4*	
C4_06910W_A	*SAP4*	
C4_07140W_A	*IHD1*	
C5_01070C_A	*ALS2*	
CHT2	*C7_01030C_A*	
MRV2	*DBP7*	
GPX2	*ENA21*	

Table AIII.3 Continuation

Wor1 (73 target genes)		
HGC1	*SIM1*	*MNN1*
HAC1	*HNM1*	*GAP1*
AOX2	*RTA2*	*SUT1*
C3_02390W_A	*PMC1*	*GRF10*
CHT2	*ZCF1*	*C6_00290W_A*
CR_06650C_A	*C3_02660W_A*	*NOP5*
C1_00160C_A	*C3_03460C_A*	*CAN2*
HGT2	*C3_04730C_A*	*SAP6*
GAL7	*C3_05160C_A*	*SAP5*
DIP5	*WOR2*	*MDR1*
PUT4	*RHB1*	*SAP4*
C1_04010C_A	*MNN14*	*C7_01690W_A*
BRG1	*AHR1*	*CUP9*
RPS27A	*ALS6*	*ENA21*
UME6	*C3_07550C_A*	*C7_04070C_A*
PBR1	*WOR4*	*C7_04080C_A*
RME1	*PTC8*	*BMT3*
C1_09330W_A	*HWP1*	*PTC2*
WOR1	*PGA31*	*NCS2*
GLY1	*MNN4*	*WOR3*
C1_10580C_A	*NOP1*	*GNP3*
FCY23	*CZF1*	*INO1*
SAM2	*GIT2*	*PGA11*
PHO84	*CYC3*	
HYR1	*PTH2*	

Tec1 (80 target genes)			
HGT1	*CHT2*	*IFD6*	*ATP9*
HGT2	*GRF10*	*HAC1*	*NAD2*
DIP5	*C6_00290W_A*	*RME1*	*NAD4*
BRG1	*GPD2*	*HYR1*	*IFE2*
UME6	*SAP6*	*SOD6*	*C4_07150W_A*
PBR1	*SAP5*	*SOD5*	
AOX2	*SAP4*	*RPL33*	
SWE1	*ALS1*	*MET10*	
PHO84	*IHD1*	*PES1*	
SIM1	*C7_00630C_A*	*ERG6*	
RIM101	*CUP9*	*C3_03460C_A*	
NDT80	*SFL1*	*AAP1*	
C2_02390W_A	*SOK1*	*C4_01220C_A*	
GFA1	*CR_06650C_A*	*PHO89*	
C3_02390W_A	*ALS3*	*PGA31*	
ADE2	*CR_07080W_A*	*PHR1*	
TEC1	*CR_07170W_A*	*CSA2*	
C3_04730C_A	*ECM22*	*ZRT1*	
AHR1	*CYS3*	*PRA1*	
CRH11	*INO1*	*SFL2*	
ECE1	*CR_10470C_A*	*PGA25*	
HWP1	*HMX1*	*GNP1*	
MNN22	*C1_01510W_A*	*ALS5*	
CZF1	*SCW4*	*C7_01510W_A*	
SUT1	*EXG2*	*ENA21*	

Rfx2 (6 target genes)
BRG1
NDT80
HYR1
ECE1
HWP1
ALS3

Table AIII.3 Continuation

Ndt80 (206 target genes)							
HGC1	*WOR1*	*C2_09460C_A*	*C3_07550C_A*	*C5_02590C_A*	*C7_00630C_A*	*CLG1*	*GIT2*
C1_01130W_A	*TPK1*	*C2_09880C_A*	*WOR4*	*PUT1*	*C7_01030C_A*	*ECM22*	*C5_01070C_A*
C1_01510W_A	*GLY1*	*SMM1*	*PTC8*	*MNN1*	*CUP9*	*YHB5*	*SUT1*
HGT1	*C1_10580C_A*	*CYC1*	*AGP2*	*GAP1*	*ENA21*	*ELF1*	*MRV2*
HGT2	*FCY23*	*ATO1*	*CRD2*	*GAP6*	*ENP2*	*CR_09920W_A*	*FCA1*
GAL7	*C1_11080W_A*	*FLC1*	*C4_01300W_A*	*SUI1*	*C7_03590C_A*	*CR_10470C_A*	*C7_01510W_A*
SCW4	*C1_11280W_A*	*UTP20*	*ERG251*	*CHT2*	*COX2*	*HMX1*	*C7_01700W_A*
DIP5	*SAM2*	*C3_01560W_A*	*PHO89*	*MRV4*	*NAD6*	*EXG2*	*CR_01710W_A*
PDE2	*PHO84*	*ERG6*	*C4_02720C_A*	*MRV6*	*NAD1*	*UPC2*	*ERG25*
C1_04010C_A	*FAD3*	*ZCF1*	*DOT4*	*PGA37*	*ATP9*	*TPO3*	*SLP3*
IFD6	*HYR1*	*C3_02660W_A*	*ECE1*	*SFL2*	*NAD2*	*MET3*	*UGA3*
BRG1	*SIM1*	*C3_03460C_A*	*HWP1*	*C5_04850W_A*	*NAD3*	*SOD5*	*INO1*
MTM1	*FTR2*	*C3_03470W_A*	*PGA31*	*GRF10*	*NAD4L*	*C2_00750W_A*	*C1_05220C_A*
PRN1	*NDT80*	*PTR22*	*C4_04520W_A*	*C6_00290W_A*	*NAD5*	*HNM1*	*C1_09710C_A*
C1_05950C_A	*HGT7*	*SAP9*	*PHR1*	*GNP1*	*NAD4*	*TBF1*	*C3_02630C_A*
PGA45	*C2_01760C_A*	*ADE2*	*HAP41*	*GPX2*	*COX3B*	*GDH2*	*MRV8*
HAC1	*ROD1*	*TEC1*	*CFL2*	*GPD2*	*CR_00280C_A*	*PES1*	*DRS1*
UME6	*C2_02390W_A*	*C3_04730C_A*	*MNN4*	*C6_02100W_A*	*CR_00290W_A*	*SKN1*	
C1_06540C_A	*SCH9*	*C3_05160C_A*	*GUT2*	*SAP6*	*BMT3*	*CAR2*	
C1_07220W_A	*KTI11*	*WOR2*	*CZF1*	*MRT4*	*AQY1*	*C4_01220C_A*	
RME1	*C2_05750W_A*	*RHB1*	*CSA2*	*SAP5*	*CR_03480W_A*	*C4_02770C_A*	
PHO100	*C2_05770W_A*	*C3_05450C_A*	*C5_00510W_A*	*MDR1*	*WOR3*	*CRH11*	
MNN47	*CNT*	*MNN14*	*CYC3*	*SAP4*	*CR_04110W_A*	*C4_03460C_A*	
AOX2	*GIT1*	*AHR1*	*GPR1*	*ALS1*	*SFL1*	*MNN22*	
C1_09330W_A	*SIT1*	*ALS6*	*FYV5*	*HGT10*	*SOK1*	*GAP5*	
SWE1	*C2_08910C_A*	*ALS7*	*HIP1*	*IHD1*	*ALS3*	*ZRT1*	
DBP3	*MET10*	*TRY6*	*PTH2*	*HGT12*	*CR_07080W_A*	*PRA1*	

Table AIII.3 Continuation

Efg1 (201 target genes)							
C1_01510W_A	*KTI11*	*C4_02720C_A*	*C7_01910C_A*	*RPS27A*	*RAP1*	*TOP1*	*C1_07790C_A*
HGT1	*RPL33*	*ECE1*	*CUP9*	*PRN3*	*C2_10160W_A*	*CAN1*	*C1_08610C_A*
HGT2	*GIT1*	*PGA31*	*C7_03230C_A*	*HAC1*	*NCE103*	*GPD2*	*C1_11000C_A*
GAL7	*PHO86*	*AGP3*	*WOR3*	*UPC2*	*CMK1*	*POP4*	*C3_01150C_A*
DIP5	*SIT1*	*HAP41*	*RNR3*	*TPO3*	*RPS19A*	*SAP5*	*C3_01430W_A*
BRG1	*MET10*	*CFL2*	*SFL1*	*MSS116*	*C3_05900W_A*	*NOG2*	*C3_01680C_A*
UME6	*C2_09880C_A*	*GUT2*	*SOK1*	*MNN47*	*GDA1*	*HGT10*	*C3_04510W_A*
RME1	*SMM1*	*CZF1*	*ALS3*	*AOX2*	*NSA2*	*ALS2*	*C3_05140C_A*
C1_09330W_A	*ATO1*	*C5_00510W_A*	*CR_07080W_A*	*TPK1*	*C3_06390W_A*	*FRP2*	*C4_06370C_A*
SWE1	*FLC1*	*PTH2*	*CR_07170W_A*	*C1_11050W_A*	*TRY6*	*HGT12*	*C5_00790C_A*
WOR1	*PMC1*	*C5_02280C_A*	*ECM22*	*PET127*	*ZFU2*	*NOP15*	*C6_01040C_A*
GLY1	*C3_01560W_A*	*PDE1*	*YHB5*	*SAM2*	*UBI4*	*C7_01510W_A*	*C7_02370W_A*
C1_10580C_A	*ZCF1*	*MNN1*	*SLP3*	*MET3*	*PGA10*	*ENA21*	
FCY23	*C3_02660W_A*	*SUT1*	*INO1*	*FTR2*	*AGP2*	*C7_04070C_A*	
C1_11080W_A	*PTR22*	*SFL2*	*POP3*	*SOD5*	*C4_01300W_A*	*CR_00280C_A*	
PHO84	*ADE2*	*GRF10*	*IFE2*	*MXR1*	*RPC53*	*CR_00290W_A*	
FEN1	*TEC1*	*C6_00290W_A*	*C1_00270W_A*	*HPT1*	*HWP1*	*ERG25*	
AGC1	*C3_04730C_A*	*GPX2*	*ENA2*	*SUR2*	*PHR1*	*CR_02440W_A*	
HYR1	*C3_05160C_A*	*CAN2*	*HGC1*	*TBF1*	*MNN22*	*DBP2*	
SIM1	*WOR2*	*FAD2*	*RRS1*	*KTR2*	*MNN4*	*DRE2*	
RIM101	*RHB1*	*C6_02100W_A*	*ZCF17*	*ECM17*	*C4_07260W_A*	*AQY1*	
NDT80	*C3_05450C_A*	*SAP6*	*SET6*	*SCP1*	*SEC14*	*SRP40*	
C2_00750W_A	*AHR1*	*SAP4*	*PUT4*	*IMH3*	*GIT2*	*QDR1*	
HGT7	*ALS6*	*ALS1*	*PDE2*	*C2_06520C_A*	*PUT1*	*RPL7*	
CHA1	*ALS7*	*IHD1*	*ELA1*	*NOC4*	*CHT2*	*CR_06650C_A*	
C2_02390W_A	*C3_07550C_A*	*C7_00630C_A*	*C1_04040C_A*	*GDH2*	*MRV2*	*YHB4*	
SCH9	*WOR4*	*C7_01690W_A*	*RRP6*	*UTP15*	*PGA37*	*C1_05540C_A*	

Table AIII.3 Continuation

Brg1 (78 target genes)			
HMT1	*C3_03470W_A*	*PTH2*	*ECE1*
HGT1	*PTR22*	*CHT2*	*HWP1*
HGT2	*ADE2*	*SFL2*	*ALS3*
GAL7	*TEC1*	*GRF10*	
DIP5	*C3_04730C_A*	*C6_00290W_A*	
IFD6	*C3_05160C_A*	*TOP1*	
BRG1	*WOR2*	*CAN1*	
UME6	*RHB1*	*FAD2*	
RME1	*C3_05450C_A*	*GPD2*	
WOR1	*AHR1*	*MDR1*	
C1_10580C_A	*ALS6*	*SAP4*	
FCY23	*ALS7*	*ALS1*	
C1_11080W_A	*C3_07550C_A*	*HGT10*	
FAD3	*WOR4*	*IHD1*	
SIM1	*AGP2*	*HGT12*	
RIM101	*CRD2*	*C7_00630C_A*	
HGT7	*C4_02720C_A*	*CUP9*	
SCH9	*PGA31*	*C7_02280W_A*	
MET10	*AGP3*	*WOR3*	
C2_09460C_A	*HAP41*	*CR_04680C_A*	
C2_09880C_A	*GUT2*	*RNR3*	
SMM1	*CZF1*	*SFL1*	
ZCF1	*C5_00510W_A*	*SOK1*	
C3_02660W_A	*CYC3*	*ECM22*	
C3_03460C_A	*HIP1*	*C1_01510W_A*	

Flo8 (10 target genes)
BRG1
NDT80
HGC1
HYR1
ECE1
HWP1
SAP5
ALS1
IHD1
ALS3

Aft2 (8 target genes)
HYR1
ECE1
HWP1
ALS3
WOR1
SIT1
MRS4

Table AIII.4 List of genes found to be altered (up- and down-regulated) in *Candida albicans* biofilms grown 24 h in RMPI supplemented with progesterone. Genes whose expression was found to increase or decrease (above or below 2-fold) in *C. albicans* SC5314 biofilms grown 24 h in RPMI (pH 4) containing 2 μM of progesterone, in comparison with the transcript levels registered in planktonic cells grown in hormone-free medium, were selected and are herein shown. The biological function indicated is based on the information available at Candida Genome Database.

Up-regulated genes		
Gene	**Biological function**	**mRNA biofilm / mRNA planktonic**
INO1	Inositol-1-phosphate synthase	47.93
ALS1	Cell-surface adhesin	22.76
PGA45	Putative GPI-anchored cell wall protein	20.17
HGT2	Putative MFS glucose transporter	15.45
ECE1	Candidalysin	14.68
CAN2	Basic amino acid permease	14.33
ENA2	Putative sodium transporter	13.52
HGT1	High-affinity MFS glucose transporter	13.45
GAP1	Amino acid permease	13.41
SOD5	Cu-containing superoxide dismutase	13.30
C1_13130C_A	Putative histidine permease	13.23
ERG6	Protein involved in ergosterol biosynthesis	12.65
MUP1	Putative high affinity methionine permease	11.73
PHR1	Cell surface glycosidase	11.18
DIP5	Dicarboxylic amino acid permease	10.19
PGA31	Cell wall protein involved in cellular response to drug	10.18
SKN1	Protein with a role in beta-1,6-glucan synthesis	10.09
HMX1	Heme oxygenase	9.05
IHD1	GPI-anchored protein	8.52
GDH2	Putative NAD-specific glutamate dehydrogenase	8.48
MRV8	Unknown function	8.24
SIT1	Transporter of ferrichrome siderophores	8.11
ALS3	Cell wall adhesin	7.68
ALS2	ALS family protein	7.62
ATO1	Putative transmembrane protein	7.62
PHO84	High-affinity phosphate transporter	7.56
SAP5	Biofilm-specific aspartyl protease	7.50
HIP1	Amino acid permease	7.30
C4_02930W_A	Unknown function	6.85
HYR1	Hyphal cell wall protein involved in biological adhesion	6.82
C2_10160W_A	Secreted protein	6.38
PRA1	Cell surface protein that sequesters zinc from host tissue	6.23
PUT2	Putative delta-1-pyrroline-5-carboxylate dehydrogenase	6.08
RIM101	Transcription factor	5.87
GAP6	Broad-specificity amino acid permease	5.86
CAR1	Arginase	5.60
SAP6	Biofilm-specific aspartyl protease	5.58
MET3	ATP sulfurlyase	5.51
MTM1	Predicted metallochaperone activity	5.45
DED1	Predicted ATP-dependent RNA helicase	5.40
ALP1	Cystine transporter	5.36
GNP1	Putative asparagine/glutamine permease;	5.31
UGA1	Putative GABA transaminase	5.26
MRV2	Unknown function	5.23
CR_09920W_A	Predicted amino acid transport domain	5.23

AGP2	Amino acid permease	5.16
SAP9	Secreted aspartyl protease	5.13
AGP3	Putative serine transporter	5.12
GIT2	Putative glycerophosphoinositol permease	5.04
C3_03460C_A	Unknown function	5.03
RTA2	Flippase involved in sphingolipid long chain base release	5.01
C2_04080W_A	Unknown function	4.87
FAD2	Delta-12 fatty acid desaturase	4.85
NCE103	Carbonic anhydrase	4.80
CYS3	Cystathionine gamma-lyase	4.78
FAD3	Omega-3 fatty acid desaturase	4.78
C3_02870C_A	Unknown function	4.74
AAP1	Putative amino acid permease	4.72
HGT12	Glucose, fructose and mannose transporter	4.68
C1_01510W_A	Unknown function	4.67
HGT10	Glycerol permease involved in glycerol uptake	4.60
CAN1	Basic amino acid permease	4.60
orf19.3338	Unknown function	4.57
C7_02280W_A	Unknown function	4.54
CMK1	Putative calcium/calmodulin-dependent protein kinase II	4.53
PUT1	Putative proline oxidase	4.48
EXG2	GPI-anchored cell wall protein	4.35
PGA25	Putative GPI-anchored adhesin-like protein	4.33
C1_07220W_A	Unknown function	4.32
PRO3	Delta 1-pyrroline-5-carboxylate reductase	4.27
C7_04070C_A	Unknown function	4.23
ZCF17	Putative Zn(II)2Cys6 transcription factor	4.21
C3_01290W_A	Unknown function	4.20
SLP3	Putative cation conductance protein	4.17
PHO89	Putative phosphate permease	4.17
SAP4	Secreted aspartyl proteinase	4.16
GUT2	Glycerol-3-phosphate dehydrogenase	4.15
CDA2	Putative chitin deacetylase	4.13
YHB5	Flavohemoglobin-related protein	4.08
CSA2	Extracellular protein involved in iron assimilation	4.07
GLY1	L-threonine aldolase	4.05
C6_02100W_A	Unknown function	4.02
CRD2	Metallothionein	3.98
QDR1	Putative antibiotic resistance transporter	3.96
FRP2	Putative ferric reductase	3.79
DBP2	Putative ATP-dependent RNA helicase	3.77
HEM3	Hydroxymethylbilane synthase	3.70
PTR22	Oligopeptide transporter involved in uptake of peptides	3.64
SOK1	Protein kinase	3.63
PMC1	Vacuolar calcium P-type ATPase	3.63
C5_01070C_A	Unknown function	3.63
MSS116	Putative DEAD-box protein	3.63
DOG1	Putative 2-deoxyglucose-6-phosphatase	3.60
PTH2	Putative cAMP-independent regulatory protein	3.60
HWP1	Hyphal cell wall protein	3.60
CHA1	Putative catabolic ser/thr dehydratase	3.54
TES1	Putative acyl-CoA thioesterase	3.54
HAP41	Putative Hap4-like transcription factor	3.50
MAK16	Putative constituent of 66S pre-ribosomal particles	3.49
C4_00250W_A	Unknown function	3.48
FCA1	Cytosine deaminase	3.42
C4_02770C_A	Predicted ATP, magnesium and ion binding activity	3.41
C1_11080W_A	Putative 6-phosphofructo-2-kinase	3.40

AOX2	Alternative oxidase	3.39
C1_12470W_A	Unknown function	3.32
SCW4	Putative cell wall protein	3.30
GAP5	General amino acid permease	3.29
NOG2	Putative nucleolar GTPase	3.29
DAO2	Putative D-aminoacid oxidase	3.27
CHT2	GPI-linked chitinase	3.27
ZCF1	Zn(II)2Cys6 transcription factor	3.25
C3_01680C_A	Predicted potassium ion transporter	3.23
C7_02260W_A	Unknown function	3.23
TRY6	Helix-loop-helix transcription factor	3.22
TEC1	Transcription factor required for biofilm formation	3.20
MNN14	Predicted alpha-1,3-mannosyltransferase activity	3.20
C2_09880C_A	Unknown function	3.20
NMD3	Putative nonsense-mediated mRNA decay protein	3.20
SFL1	Transcription factor	3.20
PHO88	Protein with a role in phosphate transport	3.20
SUR2	Putative ceramide hydroxylase	3.19
MAK21	Putative 66S pre-ribosomal particle subunit	3.19
C2_05980C_A	Putative acyl-CoA hydrolase activity	3.17
UTP21	Putative U3 snoRNP protein	3.16
CDR1	Multidrug transporter of ABC superfamily	3.15
CDR2	Multidrug transporter of ABC superfamily	3.15
CR_06650C_A	Unknown function	3.15
CIS2	Putative role in regulation of biogenesis of the cell wall	3.14
ZRT1	Putative zinc transporter	3.11
CR_00290W_A	Predicted component of the protein phosphatase type 2A	3.10
GPD2	Surface protein similar to glycerol 3-P dehydrogenase	3.10
C3_03470W_A	Succinate semialdehyde dehydrogenase	3.09
C1_00510W_A	Putative methyl-2oxobutanoate hydroxymethyl transferase	3.09
ECM22	Zn(II)2Cys6 transcription factor	3.09
C1_03790C_A	Putative peptidyl-prolyl cis-trans isomerase	3.06
AUR1	Inositol phosphoryl ceramide synthase	3.06
CUP9	Transcription factor	3.03
NOP1	Nucleolar protein	3.02
PAD1	Putative phenylacrylic acid decarboxylase	2.98
NPL3	Putative RNA-binding protein	2.98
C4_07040W_A	Putative cytochrome P450 protein	2.97
C2_06530W_A	Predicted DNA replication licensing factor	2.96
C2_06890C_A	Putative oxidoreductase	2.96
ELF1	Putative mRNA export protein	2.94
KTI11	Zn-ribbon protein	2.94
AQY1	Aquaporin water channel	2.93
ECM17	Putative sulfite reductase beta subunit	2.92
MNN4	Regulator of mannosyl phosphorylation	2.92
NTH1	Neutral trehalase	2.92
NSA2	Putative protein constituent of 66S pre-ribosomal particles	2.92
C3_05160C_A	Predicted role in assembly of pre-ribosome	2.91
FTR2	High-affinity iron permease	2.91
UTP20	Putative snoRNA-binding protein	2.91
DBP3	Putative ATP-dependent RNA helicase	2.90
HAC1	bZIP transcription factor	2.89
UTP13	Putative U3 snoRNA-associated protein	2.87
RPS27A	Ribosomal protein S27	2.85
ABP140	Predicted actin-binding protein	2.85
C1_00160C_A	Putative nucleolar protein	2.84
ALS5	ALS family adhesin	2.83
RHD3	GPI-anchored yeast-associated cell wall protein	2.83

ANT1	Peroxisomal adenine nucleotide transporter	2.83
GAR1	Putative pseudo uridylase complex protein	2.83
C3_02660W_A	Unknown function	2.83
SRP40	Putative chaperone of ribonucleoprotein particles	2.82
CR_05450C_A	Unknown function	2.82
CR_04680C_A	Unknown function	2.80
C3_06390W_A	Unknown function	2.80
ENA21	Predicted P-type ATPase sodium pump	2.78
CAR2	Ornithine aminotransferase	2.78
C2_05160C_A	Predicted translation repressor activity	2.77
SEN2	Putative tRNA splicing endonuclease subunit	2.77
UME6	Zn(II)2Cys6 transcription factor	2.76
NOP5	Predicted involvement in pre-rRNA process	2.76
DBP7	Putative ATP-dependent RNA helicase	2.75
orf19.3479	Predicted ORF merged with *C6_02350C_A*	2.75
C7_02370W_A	Protein with chitin synthesis regulation	2.75
C2_00130W_A	Unknown function	2.74
C1_04040C_A	Putative U3-containing 90S preribosome subunit	2.74
C4_00810C_A	Predicted serine-type endopeptidase activity	2.74
C1_09330W_A	Putative ribosome-associated protein	2.74
C6_01780C_A	Predicted chloride transporter	2.72
HPT1	Putative hypoxanthine-guanine phosphoribosyl transferase	2.72
WOR4	Predicted C2H2 zinc finger protein	2.72
C2_02650C_A	Predicted siroheme synthase middle domains	2.71
TAZ1	Putative lyso-phosphatidylcholine acyltransferase	2.71
PHO100	Putative inducible acid phosphatase	2.70
ECM1	Putative pre-ribosomal factor	2.70
CLG1	Putative cyclin-like protein	2.70
C5_03920C_A	Putative rRNA processing protein	2.69
C3_06680C_A	Unknown function	2.68
C7_04090C_A	Predicted mitochondrial cardiolipin-specific phospholipase	2.68
SET6	Unknown function	2.67
HIS3	Imidazole glycerol-phosphate dehydratase	2.67
C3_02630C_A	Unknown function	2.66
MET10	Sulfite reductase	2.66
NOP15	Nucleolar ribosome biogenesis factor	2.66
C5_04500W_A	Unknown function	2.65
IMH3	Inosine monophosphate (IMP) dehydrogenase	2.63
C2_04120C_A	Predicted rRNA binding activity	2.63
PTC2	Protein phosphatase of the Type 2C-related family	2.62
PET127	Protein with a predicted role in processing of RNAs	2.62
POT1	Putative peroxisomal 3-oxoacyl CoAthiolase	2.61
C4_07020C_A	Unknown function	2.61
KRE30	YEF3-subfamily ABC family protein	2.61
C6_00290W_A	Unknown function	2.61
SMM1	Putative dihydrouridine synthase	2.61
ARX1	Putative ribosomal large subunit biogenesis protein	2.61
UTP4	Putative U3 snoRNA-associated protein	2.60
SAM2	S-adenosylmethionine synthetase	2.60
GAL7	Putative galactose-1-phoshphate uridyl transferase	2.60
RLI1	Member of RNase L inhibitor (RLI) subfamily of ABC family	2.60
UPC2	Zn2-Cys6 transcription factor;	2.60
C1_09710C_A	Predicted role in endonucleolytic cleavage	2.60
C1_04710C_A	Predicted nucleolar protein	2.59
C3_04510W_A	Unknown function	2.59
C2_06520C_A	Putative thiamine transmembrane transporter	2.59
UAP1	UDP-N-acetylglucosamine pyrophosphorylase	2.59
REP1	Negative regulator of *MDR1* transcription	2.59

AGC1	Putative mitochondrial carrier protein	2.59
HGT7	Putative MFS glucose transporter	2.58
RHD2	Predicted nucleocapsid-like protein	2.57
C1_04510W_A	Putative transcription factor with bZIP DNA-binding motif	2.57
SOD6	Copper-containing superoxide dismutase	2.57
MDR1	Plasma membrane multidrug efflux pump	2.57
RPF2	Putative pre-rRNA processing protein	2.57
GDA1	Golgi membrane GDPase	2.56
HNM1	Putative choline/ethanolamine transporter	2.55
C1_10950C_A	Putative serine/threonine-protein kinase	2.55
KTR2	Mannosyl transferase	2.54
C7_00490C_A	Putative AdoMet-dependent proline methyltransferase	2.54
C3_02040C_A	Predicted nuclear protein	2.54
CRH11	GPI-anchored cell wall transglycosylase	2.53
ARO9	Aromatic transaminase	2.53
YMX6	Putative NADH dehydrogenase	2.53
CIC1	Putative proteasome-interacting protein	2.53
RRP8	Ribosomal protein	2.53
TOP1	DNA topoisomerase I	2.52
ECM18	Unknown function	2.52
BMT3	Beta-mannosyl transferase	2.52
SPB1	Putative AdoMet-dependent methyltransferase	2.52
RHB1	Putative small G protein from the Ras superfamily	2.51
PBR1	Protein required for adhesion and biofilm formation	2.51
MAL31	Putative high-affinity maltose transporter	2.51
C2_06440C_A	Putative peroxisomal membrane proteins	2.50
UGA3	Zn(II)2Cys6 transcription factor	2.50
ZCF3	Zn(II)2Cys6 domain transcription factor	2.50
C4_07150W_A	bZIP transcription factor	2.50
PES1	Putative mRNA-binding protein	2.49
C6_01040C_A	ATP-dependent RNA helicase	2.49
REI1	Putative cytoplasmic pre-60S factor	2.48
CDC13	Essential protein involved in telomere maintenance	2.48
C4_06210C_A	Putative GTPase	2.46
SIK1	Putative U3 snoRNP protein	2.46
RPL7	Ribosomal protein L7	2.46
C1_02450C_A	Predicted role in cellular bud site selection	2.46
SAM4	Putative methyltransferase	2.46
CR_08940W_A	Predicted tRNA (adenine-N1-)-methyltransferase activity	2.46
C2_02580W_A	Predicted MFS membrane transporter	2.46
ATO5	Putative fungal-specific transmembrane protein	2.45
SGE1	Putative multidrug resistance factor	2.45
HMT1	Major type I protein arginine methyltransferase (PRMT)	2.45
DAP2	Putative dipeptidyl aminopeptidase	2.44
C3_01690W_A	Putative role in positive regulation of catalytic activity	2.44
DIP2	Putative small ribonucleoprotein complex	2.44
CR_03320C_A	Unknown function	2.44
NOP4	Putative nucleolar protein	2.44
C1_06820W_A	Unknown function	2.44
C4_07140W_A	Predicted mitochondrial intermembrane space protein	2.43
C6_02290C_A	Putative flavodoxin	2.43
C3_07430W_A	Predicted acid phosphatase activity	2.43
FAS2	Alpha subunit of fatty-acid synthase	2.43
C1_00540C_A	Protein with a Bul1 domain	2.42
TRM9	Putative tRNA methyltransferase	2.42
AAH1	Adenine deaminase	2.42
CR_03540W_A	Unknown function	2.42
OAC1	Putative mitochondrial inner membrane transporter	2.41

PUT4	Putative proline permease	2.41
RAP1	Transcription factor	2.41
C5_03970W_A	Alpha/beta-Hydrolase superfamily protein	2.41
C3_05280C_A	Predicted fatty acid acyl transferase	2.40
NOG1	Putative GTPase	2.40
UTP5	Putative U3 snoRNA-associated protein	2.40
C3_04120C_A	Unknown function	2.40
C1_05950C_A	Unknown function	2.40
C1_08770W_A	Ornithine cyclodeaminase family protein	2.40
C7_03590C_A	Putative ember of the multi-drug and toxin extrusion family	2.40
SUV3	RNA helicase	2.40
C7_02010C_A	Predicted aldehyde dehydrogenase	2.40
ASM3	Putative secreted acid sphingomyelin phosphodiesterase	2.39
GRF10	Putative homeodomain transcription factor	2.39
C3_05140C_A	Predicted tRNA (guanine-N2-)-methyltransferase activity	2.39
NOP14	Putative nucleolar protein	2.39
C4_06910W_A	PDR-subfamily ABC transporter	2.39
SCH9	Protein kinase	2.39
C1_05480C_A	Unknown function	2.38
C7_01690W_A	Predicted zinc ion binding activity	2.38
YHB4	Protein related to flavohemoglobins	2.38
PHO86	Putative endoplasmic reticulum protein	2.38
NHP2	Putative H/ACA snoRNPprotein	2.37
SIM1	Adhesin-like protein	2.37
C6_02690C_A	Unknown function	2.37
C2_08510W_A	Unknown function	2.37
C3_04380C_A	Putative U3 snoRNP protein	2.36
CR_07080W_A	Predicted constituent of 66S pre-ribosomal particles	2.36
RRP9	Ribosomal protein	2.36
C3_07550C_A	Protein required for mitochondrial ribosome biogenesis	2.35
GPR1	Plasma membrane G-protein-coupled receptor	2.35
SCP1	Putative cortical actin cytoskeleton protein	2.35
NOC2	Putative nucleolar complex protein	2.35
C6_01890C_A	Putative helicase	2.35
HBR3	Essential protein	2.35
CDS1	Unknown function	2.35
RLP24	Putative ribosomal protein	2.35
C2_02920W_A	Putative adhesin-like protein	2.35
DOT4	Protein similar to ubiquitin C-terminal hydrolase	2.35
C3_01560W_A	Predicted RNA helicase	2.35
ZCF30	Predicted Zn(II)2Cys6 transcription factor	2.35
C3_02350W_A	Predicted role in endonucleolytic cleavage	2.34
C6_02420W_A	Putative phosphatidyl glycerol phospholipase C	2.34
C4_04520W_A	Predicted enzyme activator	2.33
RHR2	Putative glycerol 3-phosphatase	2.33
C5_04960W_A	Unknown function	2.33
C7_00330C_A	Predicted single-stranded telomeric DNA binding activity	2.32
TPK1	cAMP-dependent protein kinase	2.32
CR_01510C_A	Unknown function	2.32
UTP9	Small-subunit processome protein	2.32
DRS1	Putative nucleolar protein	2.32
SDH4	Succinate dehydrogenase	2.32
C2_00530W_A	Unknown function	2.31
HGT13	Predicted sugar transporter	2.31
CR_04170W_A	Predicted rRNA (cytosine-C5-)-methyltransferase activity	2.31
ENP2	Putative nucleolar protein	2.31
C1_13370W_A	Predicted role in protein catabolic process	2.31
HGC1	Hypha-specific G1 cyclin-related protein	2.31

FAA4	Predicted acyl CoA synthase	2.30
C2_08910C_A	Protein with a metallo-dependent phosphatase domain	2.30
NDT80	Meiosis-specific transcription factor	2.30
TBF1	Essential transcription factor	2.30
MAL2	Alpha-glucosidase	2.30
C1_06140C_A	Unknown function	2.29
GNP3	Putative high-affinity glutamine permease	2.29
MAK5	Putative RNA helicase	2.29
C7_03230C_A	Unknown function	2.29
ALS6	ALS family protein	2.29
PLB2	Putative phospholipase B	2.29
C2_08830W_A	Putative role in chitin localization	2.29
C3_06130W_A	Unknown function	2.28
FLC3	Protein involved in heme uptake	2.28
C5_02590C_A	Putative mitochondrial membrane protein	2.28
C3_03130C_A	Predicted role in cell wall biogenesis	2.28
CR_03310C_A	Putative transcription factor	2.27
RIX7	Putative ATPase of the AAA family	2.27
C1_10050W_A	Unknown function	2.27
C1_09040C_A	Putative protein-histidine N-methyltransferase	2.27
CR_10470C_A	Predicted rRNA binding activity	2.26
CR_04600W_A	Unknown function	2.26
C7_03210W_A	Unknown function	2.26
C4_00860C_A	Unknown function	2.26
RPC19	Putative RNA polymerases I and III subunit AC19	2.26
SUI3	Putative translation initiation factor	2.25
OPT8	Oligopeptide transporter	2.25
C3_04260W_A	Predicted role in metal ion transport	2.25
C3_03940C_A	Unknown function	2.25
ERG25	Putative C-4 methyl sterol oxidase	2.25
RPC40	Putative RNA polymerase	2.25
ALS7	ALS family protein	2.25
FLC1	Protein involved in heme uptake	2.24
RSM22	Predicted mitochondrial small ribosomal subunit	2.24
NMA111	Putative serine protease and general molecular chaperone	2.24
CR_02430C_A	Predicted protein of rapamycin resistance	2.24
C7_01750W_A	Putative mitochondrial outer membrane protein	2.24
BTA1	Unknown function	2.23
UTP15	Small subunit (SSU) processome component	2.23
C4_02260C_A	Unknown function	2.23
C5_05360C_A	Putative steryl-beta-glucosidase activity	2.23
RPA34	Putative RNA polymerase I subunit	2.22
C2_02390W_A	Unknown function	2.22
FUN12	Translation initiation factor	2.22
HAS1	Predicted ATP-dependent RNA helicase	2.22
C3_06370C_A	Predicted role in endonucleolytic cleavage	2.22
C6_03600C_A	Putative cytochrome P450	2.22
RPF1	Predicted role in the assembly of ribosomal subunits	2.22
UTP8	Nucleolar protein involved in tRNA export from the nucleus	2.21
C6_02230W_A	Putative GTPase	2.21
C2_00280C_A	Putative pre-mRNA-splicing factor	2.21
C1_13820C_A	Unknown function	2.21
C2_09510C_A	Predicted ATP:ADP antiporter activity	2.21
MNN47	Unknown function	2.21
PPT1	Putative serine/threonine phosphatase	2.21
PZF1	C2H2 transcription factor	2.21
MIS12	Mitochondrial C1-tetrahydrofolate synthase precursor	2.21
CRZ1	Calcineurin-regulated C2H2 transcription factor	2.20

FAL1	ATP-dependent RNA helicases	2.20
SFU1	GATA-type transcription factor	2.20
C2_05810W_A	Putative role in cellular response to methylmercury	2.20
RME1	Zinc finger protein; putative meiosis control	2.20
SDA1	Predicted involvement in actin cytoskeleton organization	2.20
QDR2	Predicted MFS membrane transporter	2.20
SRP101	Signal recognition particle (SRP) receptor alpha subunit	2.20
C1_06760C_A	Putative U2 snRNP component	2.20
RAD16	Predicted protein that recognizes and binds damaged DNA	2.20
orf19.3288.1	Predicted orf merged with orf19.3288 (*NMA111*)	2.20
FCY23	Putative transporter	2.20
CNT	CNT family H(+)/nucleoside symporter	2.19
ADE4	Putative phosphoribosyl pyrophosphate amido transferase	2.19
SEO1	Protein with similarity to permeases	2.19
C3_00640W_A	Putative cysteine sulfinate decarboxylase	2.19
C4_02720C_A	Putative plasma membrane protein	2.19
C2_04000C_A	Unknown function	2.18
YOR1	Predicted ABC-type plasma membrane transporter	2.18
C1_07950C_A	Putative pre-60S pre-ribosomal particle subunit	2.18
GUA1	Putative GMP synthase,	2.18
C2_08180C_A	Predicted role in ribosomal large subunit biogenesis	2.18
CR_09740W_A	Protein involved in rRNA processing	2.18
NOC4	Putative nucleolar protein	2.18
C4_00750C_A	Unknown function	2.18
JIP5	Predicted role in biogenesis of the large ribosomal subunit	2.18
POP3	Putative nuclear RNase component	2.18
CR_01710W_A	Putative nucleolar protein	2.17
UTP18	Putative U3 snoRNA-associated protein	2.17
C1_12440W_A	Putative RNA exonuclease	2.17
AVT1	Putative vacuolar transporter	2.17
FGR50	Unknown function	2.17
ALS4	GPI-anchored adhesin	2.17
CR_00280C_A	Predicted ATPase activity	2.17
C4_06850C_A	Predicted metalloaminopeptidase activity	2.17
MRT4	Putative mRNA turnover protein	2.17
GPD1	Glycerol-3-phosphate dehydrogenase	2.17
C2_08090W_A	Unknown function	2.16
UTP22	Putative U3 snoRNP protein	2.16
RNR3	Putative ribonucleotide reductase large subunit	2.16
C1_04640W_A	Putative serine hydrolase activity	2.16
CSI2	Putative 66S pre-ribosomal particle component	2.16
C7_03850W_A	Putative nuclear transport and DNA replication mediator	2.16
C1_11000C_A	Protein with a role in nucleolar integrity	2.16
DUR35	Putative urea transporter	2.16
CR_04500C_A	Predicted oxidoreductase activity	2.16
BMS1	Putative GTPase	2.16
C3_00840C_A	Unknown function	2.15
C7_01910C_A	Unknown function	2.15
DPP3	Predicted pyrophosphate phosphatase	2.15
C2_02710C_A	Predicted role in rRNA processing	2.15
C2_05860C_A	Putative Xbp1 transcriptional repressor	2.15
C6_03670C_A	Unknown function	2.15
ICL1	Isocitrate lyase	2.15
ISA1	Putative mitochondrial iron-sulfur protein	2.15
TPO3	Putative polyamine transporter	2.15
AKR1	Ankyrin-repeat protein	2.15
C4_01300W_A	Predicted phosphoprotein phosphatase activity	2.14
GAP2	General amino acid permease	2.14

C3_05800W_A	Predicted tRNA-intron endonuclease activity	2.14
C5_01310W_A	Predicted splicing coactivator	2.14
C1_06540C_A	Exosome non-catalytic core component	2.14
RPR2	Putative ribonuclease P activity	2.14
C3_05750C_A	Unknown function	2.13
NAN1	Putative U3 snoRNP protein	2.13
ROD1	Predicted membrane protein with a role in drug tolerance	2.13
C4_01500W_A	Protein required for thiolation of uridine	2.12
C5_02800C_A	Unknown function	2.12
C1_10620W_A	Predicted pseudouridine synthase	2.12
RBR2	Cell wall protein	2.12
C2_00940W_A	Plasma membrane-associated protein	2.12
FAT1	Predicted enzyme of sphingolipid biosynthesis	2.12
SEF2	Zn(II)2Cys6 transcription factor	2.12
SAC7	Putative GTPase activating protein (GAP)	2.11
C1_11280W_A	Predicted pre-mRNA binding activity	2.11
ELP3	Predicted histone acetyltransferase	2.11
PTC8	Predicted type 2C protein phosphatase (ser/thr-specific)	2.11
TRM2	Putative tRNA methyltransferase	2.11
RPA135	Putative RNA polymerase I subunit A135	2.11
C3_02180C_A	Deoxyhypusine synthase	2.11
DBP8	Predicted ATP-dependent helicase	2.11
NOP6	Putative role in ribosomal small subunit biogenesis	2.11
CR_00790C_A	Unknown function	2.11
C7_00160C_A	Putative role in ribosomal large subunit biogenesis	2.10
NAD1	NADH dehydrogenase involved in mitochondrial respiration	2.10
C2_02540W_A	Predicted RNA binding protein	2.10
C1_01470W_A	Putative RNA binding activity	2.10
HCA4	Putative role in regulation of cell wall biogenesis	2.10
C2_00620C_A	Putative protein-lysine N-methyltransferase activity	2.09
C1_09840C_A	Protein involved in ubiquitin-mediated protein degradation	2.09
PEX22	Putative peroxin	2.09
CR_04770C_A	Unknown function	2.09
C2_09660W_A	Putative 66S pre-ribosomal particle component	2.09
CR_08470W_A	Protein involved in N-glycosylation	2.09
CHS2	Chitin synthase	2.09
RPA190	Putative RNA polymerase I subunit A190	2.08
C4_03750C_A	Predicted translation release factor activity	2.08
C7_04140C_A	Predicted unfolded protein binding activity	2.08
FAA21	Predicted acyl CoA synthetase	2.08
C1_13210C_A	Unknown function	2.08
C7_01030C_A	Predicted mRNA binding activity	2.08
C2_07610C_A	Putative role in rescue of stalled ribosome	2.08
SOF1	Predicted role in 40S ribosomal subunit biogenesis	2.07
NSA1	Putative 66S pre-ribosomal particles conmponent	2.07
C6_00110C_A	Unknown function	2.07
CR_03360W_A	Predicted role in endonucleolytic cleavage	2.07
C2_06850W_A	Predicted role in pre-rRNA processing	2.07
C5_00350C_A	Putative DnaJ-like chaperone	2.07
CR_09800C_A	Putative U3-containing small subunit processome protein	2.07
C1_02890C_A	Unknown function	2.07
C5_04850W_A	Unknown function	2.07
CR_06680C_A	Putative DNA helicase activity	2.06
NCS2	Putative cytosolic thiouridylase subunit	2.06
POP4	Putative nuclear RNase component	2.06
C4_06620C_A	Unknown function	2.06
C1_03990W_A	Predicted proteasome-mediated protein	2.06
GDE1	Glycerophosphocholine phosphodiesterase	2.06

SEC9	Protein required for secretory vesicle-membrane fusion	2.06
CR_04110W_A	Putative RNA-binding protein	2.06
C5_05090W_A	Putative role in protein mitochondrial membrane insertion	2.05
RPB8	Putative subunit of RNA polymerases I, II, and III	2.05
NAR1	Putative cytosolic iron-sulfur protein assembly protein	2.05
TPO2	Putative polyamine transport protein	2.05
TRY3	RING-finger transcription factor	2.05
PDE1	Low affinity cyclic nucleotide phosphodiesterase	2.05
ADE2	Phosphoribosyl amino imadazole carboxylase	2.05
CDC34	Putative ubiquitin-protein ligase	2.05
IDI1	Predicted isopentenyl-diphosphate delta-isomerase activity	2.05
C1_07960W_A	Predicted nucleic acid binding protein	2.04
C2_04280W_A	Putative protein serine/threonine/tyrosine kinase activity	2.04
C3_06490W_A	Unknown function	2.04
C1_06800W_A	Predicted RNA pyrophosphohydrolase	2.04
C2_09460C_A	Predicted Gal4-like DNA-binding transcription factor	2.04
CR_01600C_A	Predicted RNA binding and ribonuclease activity	2.03
CR_01950W_A	Predicted RNA polymerase I activity	2.03
RPS3	Ribosomal protein S3	2.03
C4_05010W_A	Protein with a predicted role in ribosome biogenesis	2.03
CR_02440W_A	Unknown function	2.03
PEL1	Predicted enzyme of phospholipid biosynthesis	2.03
CR_07900C_A	Unknown function	2.02
RAD6	Unknown function	2.02
CR_06040W_A	Unknown function	2.02
CR_07690W_A	Unknown function	2.02
CR_10230W_A	Putative histone acetyltransferase activity	2.02
C1_14560C_A	Predicted retroviral reverse transcriptase protein	2.02
ENP1	Protein required for pre-rRNA processing	2.02
C2_05750W_A	Predicted rRNA binding activity	2.01
NEP1	Putative rRNA (pseudouridine) methyltransferase activity	2.01
C4_02170C_A	Putative lysophospholipase activity	2.01
ASC1	40S ribosomal subunit	2.01
SRD1	Sur7 family protein with PalI-like domains	2.01
C1_12760W_A	Putative nuclear export protein	2.01
CR_05550C_A	Putative RNA polymerase III activity	2.01
GNA1	Glucosamine-6-phosphate acetyltransferase	2.01
MTG1	Putative mitochondrial GTPase	2.01
DCK1	Putative guanine nucleotide exchange factor	2.01
C4_06790W_A	Putative role in biogenesis of RNA pol II and pol III	2.01
C7_00630C_A	Unknown function	2.00
PWP2	Putative 90S pre-ribosomal component	2.00
SAS10	Putative U3-containing small subunit processome subunit	2.00
OLE1	Fatty acid desaturase	2.00
CR_03480W_A	Unknown function	2.00
CR_10410C_A	Predicted role in pre-18S rRNA processing	2.00
SUI1	Putative translation initiation factor	2.00

Down-regulated genes

Gene	Biological function	$\frac{\text{mRNA}_{\text{biofilm}}}{\text{mRNA}_{\text{planktonic}}}$
DUR3	High affinity spermidine transporter	0.05
C1_07160C_A	Protein conserved among the CTG-clade	0.09
MEP2	Ammonium permease	0.11
CCP1	Putative Cytochrome-c peroxidase	0.12
UCF1	Predicted involvement in generation of energy	0.12

JEN2	Dicarboxylic acid transporter	0.12
RSD1	Predicted short chain dehydrogenase	0.13
CWH8	Putative dolichyl pyrophosphate (Dol-P-P) phosphatase	0.14
C1_01630W_A	Putative mitochondrial complex I associated protein	0.15
C2_01660C_A	Unknown function	0.16
HSP30	Putative heat shock protein	0.16
ADH2	Alcohol dehydrogenase;	0.16
PGA13	GPI-anchored cell wall protein involved in cell wall synthesis	0.16
FDH1	Formate dehydrogenase	0.16
C5_03710C_A	Unknown function	0.16
DAK2	Putative dihydroxyacetone kinase	0.16
C2_00180C_A	Predicted uricase	0.16
HAK1	Putative potassium transporter	0.17
LAP3	Putative aminopeptidase	0.17
GRE3	Putative D-xylose reductase	0.17
ZRT3	Unknown function	0.18
MMD1	Possibly required for transamination of isoleucine	0.18
ARO10	Aromatic decarboxylase	0.18
C5_03770C_A	Putative formate dehydrogenase	0.19
C1_10360C_A	Unknown function	0.19
C1_14090W_A	Putative role in chaperone-mediated protein assembly	0.19
C2_01540W_A	Predicted sulfonate dioxygenase	0.19
CRZ2	C2H2 transcription factor	0.19
C2_00860C_A	Unknown function	0.19
SOD4	Cu-containing superoxide dismutase	0.19
C5_00100C_A	Unknown function	0.20
CR_09070C_A	Unknown function	0.20
PDC11	Pyruvate decarboxylase	0.20
HSP21	Heat shock protein	0.21
ZRT2	Zinc transporter	0.21
HSP31	Putative heat shock protein	0.21
C1_10280C_A	Unknown function	0.21
YKE2	Possible heterohexamericGim/prefoldin protein	0.22
ARG3	Putative ornithine carbamoyltransferase	0.22
HGT18	Putative glucose transporter of major facilitator superfamily	0.22
ENO1	Enolase involved in glycolysis and gluconeogenesis	0.22
C1_03510C_A	Unknown function	0.23
C1_12180C_A	Unknown function	0.23
HEM13	Coproporphyrinogen III oxidase	0.24
PEP1	Type I transmembrane sorting receptor	0.24
NGT1	N-acetylglucosamine (GlcNAc)-specifictransporter	0.24
MDH1-1	Predicted malate dehydrogenase precursor	0.24
PSA2	Mannose-1-phosphate guanyltransferase	0.24
GLK1	Putative glucokinase	0.24
FMP45	Predicted membrane protein induced during mating	0.24
TDH3	NAD-linked glyceraldehyde-3-phosphate dehydrogenase	0.25
C6_02030C_A	Unknown function	0.25
CTN1	Carnitine acetyl transferase	0.25
C2_09820W_A	Unknown function	0.25
ZSF1	Predicted mRNA-binding protein	0.25
C7_03560W_A	Unknown function	0.25
TPS3	Predicted trehalose-phosphate synthase	0.25
C2_05570C_A	Putative lipid raft associated protein	0.26
GLK4	Putative glucokinase	0.26
CR_01080W_A	Unknown function	0.26
FUM11	Fumarate hydratase	0.26
RVS162	Protein containing a BAR domain	0.26
C1_11920W_A	Predicted ATPase activator activity	0.26

C2_00760C_A	Unknown function	0.26
CR_06510W_A	Unknown function	0.27
C1_12720C_A	Unknown function	0.27
SOD2	Mitochondrial Mn-containing superoxide dismutase	0.27
C1_02780W_A	Predicted dienelactone hydrolase domain	0.27
HOM6	Putative homoserine dehydrogenase	0.27
C3_03570C_A	Predicted oxidoreductase activity	0.28
C7_00590W_A	Unknown function	0.28
ROT2	Alpha-glucosidase II	0.28
PGK1	Phosphoglycerate kinase	0.28
CIRT4B	Predicted involvement in DNA binding	0.28
FET99	Multicopper oxidase family protein	0.28
C1_01620C_A	Predicted oxidoreductase activity and role in metabolic process	0.28
C6_03320W_A	Unknown function	0.28
C6_03370W_A	Unknown function	0.29
FET31	Putative multicopper oxidase	0.29
C2_07070W_A	Putative quinone oxidoreductase	0.29
OSM2	Putative mitochondrial fumarate reductase	0.29
MDH1	Mitochondrial malate dehydrogenase	0.29
HSP60	Heat shock protein	0.29
IHD2	Unknown function	0.29
FBA1	Fructose-bisphosphate aldolase	0.29
CCS1	Copper chaperone	0.29
HPD1	3-hydroxypropionate dehydrogenase	0.29
CAS1	Putative transcription factor	0.29
CR_09590W_A	Predicted role in ubiquinone-6 biosynthetic process	0.30
AGO1	Putative argonaute protein involved in RNA silencing	0.30
C3_05850W_A	Predicted ubiquitin-protein transferase activity	0.30
CCN1	G1 cyclin	0.30
RGS2	Protein of RGS superfamily	0.30
PGI1	Predicted Glucose-6-phosphate isomerase	0.30
LYS12	Homoisocitrate dehydrogenase	0.30
C3_07490W_A	Predicted phosphopentomutase activity	0.30
CR_09530C_A	Predicted DDE superfamily endonuclease domain	0.30
PRX1	Thioredoxin peroxidase	0.30
C4_03370C_A	Unknown function	0.30
GVP36	BAR domain protein	0.30
C3_07470W_A	Predicted role in cell wall integrity	0.30
CR_00820C_A	Putative ribosome binding activity	0.30
ECM15	Unknown function	0.31
C1_01930W_A	Unknown function	0.31
C7_00340C_A	Putative transporter activity	0.31
C2_00770W_A	Unknown function	0.31
AMO1	Putative peroxisomal copper amine oxidase	0.31
CFL5	Predicted Ferric reductase	0.31
ADH3	Putative NAD-dependent (R,R)-butanedioldehydrogenase	0.32
EMP24	COPII-coated vesicle component	0.32
MCD1	Alpha-kleisincohesin complex subunit	0.32
C1_03100W_A	Putative lipid-binding protein	0.32
CR_06500C_A	Unknown function	0.32
C1_13190W_A	Putative mitochondrial membrane protein; unknown function	0.32
PGA38	Putative adhesin-like GPI-anchored protein	0.32
TPO4	Putative sperimidine transporter	0.32
C1_08340C_A	Sorting nexin	0.32
CR_09700W_A	Predicted membrane transporter	0.32
GND1	6-phosphogluconate dehydrogenase	0.32
RBE1	Pry family cell wall protein	0.32
C2_08100W_A	Putative mitochondrial cell death effector	0.32

PNG2	Putative peptide:N-glycanase	0.32
XYL2	D-xylulose reductase	0.32
SOD3	Cytosolic manganese-containing superoxide dismutase	0.33
C1_10310W_A	Predicted methyltransferase	0.33
C5_00330C_A	Predicted role in fermentation and protein maturation	0.33
MSH6	Predicted involvement in mismatch repair	0.33
IDP2	Isocitrate dehydrogenase	0.33
C6_04420W_A	Unknown function	0.33
C6_02660C_A	Predicted amidase activity	0.33
C5_00400C_A	Unknown function	0.33
CR_09580C_A	Putative involvement in 20S proteasome assembly	0.33
SCW11	Cell wall protein, possibly an essential gene	0.33
C4_03500C_A	Unknown function	0.33
C6_03880W_A	Predicted oxidoreductase activity	0.33
C5_00390C_A	Unknown function	0.33
MSH2	Putative DNA mismatch repair factor	0.34
SPC3	Essential protein	0.34
DLD1	Putative D-lactate dehydrogenase	0.34
NIP100	p150 subunit of dynactin	0.34
NIT3	Putative nitrilase	0.34
CR_03470W_A	Unknown function	0.34
MCR1	NADH-cytochrome-b5 reductase	0.34
CR_09140C_A	Protein with a role in directing meiotic recombination events	0.34
HSM3	Predicted role in mismatch repair	0.34
KAR9	Predicted role in mitotic spindle positioning	0.34
DUR1,2	Urea amidolyase involved in response to abiotic stimulus	0.34
OSM1	Putative flavoprotein subunit of fumarate reductase	0.35
POL30	Proliferating cell nuclear antigen (PCNA)	0.35
SER1	Putative 3-phosphoserine aminotransferase	0.35
DIT2	Monooxygenase of the cytochrome P450 family	0.35
BZZ1	Predicted role in the regulation of actin polymerization	0.35
SNO1	Predicted role in pyridoxine metabolism	0.35
C1_11120C_A	Unknown function	0.35
CDC19	Pyruvate kinase at yeast cell surface	0.35
GCN2	Translation initiation factor 2-alpha (eIF2alpha) kinase	0.35
HGT19	Putative MFS glucose/myo-inositol transporter	0.35
C4_01840C_A	Putative dienelactone hydrolase	0.35
C2_06600W_A	Predicted kinase	0.35
RIM1	Putative single-stranded DNA-binding protein	0.35
C6_01360W_A	Unknown function	0.35
C3_03680W_A	Predicted sphinganine-1-phosphate aldolase activity	0.36
CDR4	Putative ABC transporter superfamily	0.36
C1_04200C_A	Predicted structural constituent of nuclear pore activity	0.36
CSR1	Transcription factor	0.36
GPM1	Phosphoglycerate mutase	0.36
GAD1	Putative glutamate decarboxylase	0.36
CR_02800C_A	Unknown function	0.36
C5_04320C_A	RNI-like superfamily domain-containing protein	0.36
C4_04290W_A	Exocyst subunit involved in polarized growth	0.36
CR_03120W_A	Unknown function	0.37
FAV2	Adhesin-like protein	0.37
C7_03150W_A	Unknown function	0.37
C6_02490C_A	Unknown function	0.37
RIC1	Predicted guanyl-nucleotide exchange factor	0.37
C4_03050C_A	Predicted carboxypeptidase activity	0.37
C2_09590C_A	Predicted copper uptake transmembrane transporter activity	0.37
ADE17	5-Aminoimidazole-4-carboxamide ribotidetransformylase	0.37
PNP1	Purine nucleoside phosphorylase	0.37

CR_08850W_A	Unknown function	0.37
DEF1	RNA polymerase II regulator; role in filamentation	0.37
CYB2	Putative cytochrome b2 precursor	0.37
C5_02140C_A	Predicted pyridoxal 5'-phosphate synthase	0.37
C3_03760W_A	Putative diacylglycerol acyltransferase	0.37
GLO1	Putative monomeric glyoxalase I	0.37
GCY1	Aldo/keto reductase	0.37
C1_02770W_A	Unknown function	0.38
PRC2	Putative carboxypeptidase	0.38
NTF2	Putative nuclear envelope protein	0.38
DPM1	Dolichol-phosphate mannose synthase catalytic subunit	0.38
DCK2	Predicted role in filamentous growth	0.38
PCT1	Putative choline-phosphate cytidylyl transferase	0.38
C3_02340W_A	Unknown function	0.38
C1_10680C_A	Predicted lipid-binding ER protein	0.38
C1_06250W_A	Unknown function	0.38
KRE5	UDP-glucose:glycoprotein glucosyltransferase	0.38
C4_04020C_A	Unknown function	0.38
C2_09650W_A	Predicted poly(A) binding activity	0.38
GLT1	Putative glutamate synthase	0.38
C3_05840W_A	Predicted monooxygenase activity	0.38
MRF1	Putative mitochondrial respiratory protein	0.39
C3_04420W_A	Predicted ubiquitin-protein ligase	0.39
C3_06040W_A	CCCH zinc finger protein	0.39
COX9	Putative subunit VIIa of cytochrome c oxidase	0.39
C1_09520C_A	Unknown function	0.39
SAR1	Predicted involvement in ER-to-Golgi protein transport	0.39
PCL1	Cyclin homolog	0.39
RIB5	Putative riboflavin synthase	0.39
C2_03260W_A	Unknown function	0.39
FGR38	Unknown function	0.39
LYS9	Saccharopine dehydrogenase	0.39
CTA26	Putative transcription factor/activator	0.39
NUP82	Linker nucleoporin of the nuclear pore complex	0.39
C1_06340W_A	Unknown function	0.39
GAT1	GATA-type transcription factor	0.39
CR_09010C_A	Putative cystathionine gamma-synthase	0.39
C2_02790C_A	Predicted amino acid transmembrane transporter	0.39
CR_07350W_A	Predicted role in ER-dependent peroxisome organization	0.39
GWT1	Inositol acyltransferase with role in GPI anchor biosynthesis	0.39
AHP1	Alkyl hydroperoxide reductase	0.40
GCN4	bZIP transcription factor	0.40
TPM2	Putative tropomyosin isoform 2	0.40
C7_00870W_A	Putative guanine deaminase	0.40
PMT4	Protein mannosyl transferase	0.40
NAG4	Putative transporter	0.40
PGA4	GPI-anchored cell surface protein	0.40
C2_02620W_A	Putative mitochondrial protein of unknown function	0.40
C4_00420C_A	Predicted enzyme inhibitor activity	0.40
SNL1	Predicted role in protein synthesis	0.40
SOD1	Cytosolic copper- and zinc-containing superoxide dismutase	0.40
CLB4	B-type mitotic cyclin	0.40
C3_02750W_A	Protein with a ribonuclease III domain	0.40
TPS1	Trehalose-6-phosphate synthase	0.40
MNN12	Predicted alpha-1,3-mannosyltransferase activity	0.40
C6_03260W_A	Putative ribonuclease H1	0.40
ABG1	Vacuolar membrane protein	0.41
CDC55	Putative protein phosphatase type 2A regulator activity	0.41

CTA9	Protein required for normal filamentous growth	0.41
DAG7	Secretory protein	0.41
C5_03080C_A	Predicted membrane transporter	0.41
C5_02110W_A	Putative heat shock protein	0.41
SEC23	Putative GTPase-activating protein	0.41
IPP1	Putative inorganic pyrophosphatase	0.41
IQG1	Actomyosin ring component at bud neck	0.41
MED16	Putative RNA polymerase II mediator complex subunit	0.41
MEC1	Cell cycle checkpoint protein with a role in genome integrity	0.41
CBF1	Transcription factor that binds upstream of ribosomal genes	0.41
CDC10	Septin	0.41
MVD	Mevalonate diphosphate decarboxylase	0.41
USO1	Predicted role in ER to Golgi vesicle-mediated transport	0.41
PNC1	Putative nicotinamidase	0.41
PGM2	Putative phosphoglucomutase	0.41
C2_07630C_A	Putative stress protein	0.41
CR_06850C_A	Unknown function	0.42
C1_02970W_A	Predicted aminotransferase	0.42
C1_12880C_A	Predicted DNA and nucleic acid binding activity	0.42
CLN3	G1 cyclin	0.42
VMA10	Subunit G of the vacuolar H+-ATPase	0.42
BUL1	Putative role in selection of substrates for ubiquitination	0.42
LEU2	Isopropyl malate dehydrogenase	0.42
C1_11990W_A	Putative cell wall adhesin-like protein	0.42
C1_04430C_A	Unknown function	0.42
CDC3	Septin	0.42
CHC1	Clathrin heavy chain	0.42
C7_03030W_A	Unknown function	0.42
RTG1	RNA polymerase II transcription factor	0.42
C2_04480W_A	Predicted oxidoreductase activity	0.42
C1_02370C_A	Unknown function	0.42
C1_02730W_A	Unknown function	0.43
C1_09670C_A	Predicted DNA binding activity	0.43
TPD3	Subunit of protein serine/threonine phosphatase PPA2	0.43
ACH1	Acetyl-coA hydrolase	0.43
STI1	Predicted involvement in Cdc37 chaperone activity	0.43
TPK2	cAMP-dependent protein kinase catalytic subunit	0.43
IFD3	Putative aldo/keto reductase	0.43
SEC61	ER protein-translocation complex subunit	0.43
LPG20	Aldo-keto reductase family protein	0.43
C1_09980C_A	Predicted acylglycerol lipase activity	0.43
C1_00410C_A	Hexadecenal dehydrogenase	0.43
C5_02330W_A	Unknown function	0.43
LYS22	Homocitrate synthase	0.43
TIP20	Predicted role in Golgi-ER transport	0.43
C7_01650W_A	RNA polymerase III transcription initiation factor	0.43
CDC37	Chaperone for Crk1p	0.43
ERV25	Component of COPII-coated vesicles	0.43
IST2	Predicted lipid binding activity	0.43
C5_05020C_A	Dolichyl-diphosphooligosaccharide-protein glycotransferase	0.43
ATP4	Putative F0-ATP synthase subunit 4	0.43
WBP1	Putative oligosaccharyl transferase subunit	0.43
MON2	Peripheral membrane protein	0.43
C2_07640W_A	Unknown function	0.44
BET2	Putative Type II geranylgeranyl transferase beta subunit	0.44
C7_03170W_A	Unknown function	0.44
C3_07740W_A	Predicted COPI-coated vesicle	0.44
TIM22	Predicted role in protein import	0.44

RIB4	Lumazine synthase	0.44
IST1	Positive role in the multivesicular body sorting pathway	0.44
C4_00910C_A	Putative mutator-like element transposase	0.44
C2_04400W_A	Unknown function	0.44
GPH1	Putative glycogen phosphorylase	0.44
HSP104	Heat-shock protein	0.44
TLO1	Member of a family of telomere-proximal genes	0.44
BRO1	Class E vacuolar protein sorting factor	0.44
C2_01450C_A	Unknown function	0.44
CR_00600C_A	Putative role in endocytosis	0.44
CR_08560C_A	Predicted SUMO transferase activity	0.44
PMT2	Protein mannosyl transferase	0.44
BNA4	Putative kynurenine 3-monooxygenase	0.44
CR_06010W_A	Unknown function	0.44
TLO7	Member of telomere-proximal genes family; unknown function	0.44
MET28	Predicted bZIP domain-containing transcription factor	0.44
C1_10840C_A	Putative role in retrograde transport	0.45
C5_02560C_A	Predicted ATP binding and protein kinase activity	0.45
C7_01710W_A	Membrane-localized protein of unknown function	0.45
OXR1	Putative role in cellular response to oxidative stress	0.45
C2_08690C_A	Unknown function	0.45
CFL4	C-terminus similar to ferric reductases	0.45
C3_06860C_A	Putative xylose and arabinose reductase	0.45
CR_03410W_A	Unknown function	0.45
CR_09100C_A	Aldo-keto reductase	0.45
C6_02140W_A	Unknown function	0.45
UGP1	UTP-glucose-1-phosphaturidyl transferase	0.45
HBR2	Putative alanine glyoxylate aminotransferase	0.45
C3_01020W_A	Unknown function	0.45
CWH43	Putative transporter protein and role in cell wall biogenesis	0.45
MAE1	Malic enzyme	0.45
C6_03590C_A	Unknown function	0.45
YVC1	Putative vacuolar cation channel shock	0.45
ERG20	Putative farnesyl pyrophosphate synthetase	0.45
CR_00750C_A	Putative histone demethylase activity	0.45
C1_11100W_A	Putative acireductone dioxygenase	0.45
TLO34	Non-telomeric member of a family of telomere-proximal genes	0.45
SAM51	Component involved in mitochondrial protein import	0.45
MRP8	Mitochondrial ribosomal protein	0.45
XKS1	Putative xylulokinase	0.45
IFI3	Unknown function	0.45
C4_00990W_A	NRAMP metal ion transporter domain-containing protein	0.45
C1_09060C_A	Unknown function	0.45
C2_04500W_A	Predicted ATPase activity and DNA binding activity	0.45
HGT17	Putative MFS family glucose transporter	0.45
SNG4	Putative membrane transporter	0.45
PMS1	Putative DNA mismatch repair factor	0.45
C7_00350C_A	Unknown function	0.45
C1_05370C_A	Putative serine/threonine kinase	0.46
C1_09650W_A	Unknown function	0.46
TEL1	Predicted serine/threonine kinase activity	0.46
CR_02880W_A	Unknown function	0.46
C7_03730C_A	Unknown function	0.46
MAC1	Copper fist transcription factor	0.46
ATP1	ATP synthase alpha subunit	0.46
AHP2	Putative thiol-specific peroxiredoxin	0.46
C4_05860W_A	Putative tubulin binding activity	0.46
HSP90	Essential chaperone	0.46

C1_04180W_A	Predicted histone H2B	0.46
C3_06640W_A	Unknown function	0.46
SEC5	Predicted exocyst component	0.46
C1_10560C_A	Unknown function	0.46
BPH1	Unknown function	0.46
C5_04050W_A	Plasma membrane-localized protein of unknown function	0.46
GEF2	Member of the voltage chloride channel family	0.46
CHS5	Putative chitin biosynthesis protein	0.46
SNT1	Putative NAD-independent histone deacetylase	0.46
DQD1	Putative 3-dehydroquinate dehydratase	0.46
TFS1	Putative carboxypeptidase y inhibitor	0.46
C5_00080C_A	Epsilon-COP subunit of the coatomer	0.46
C7_00430W_A	Putative ferric reductase	0.46
GST2	Glutathione S transferase	0.46
C7_03370C_A	Unknown function	0.46
LHS1	Predicted Kex2p substrate	0.46
CR_00220W_A	Predicted electron carrier activity	0.46
HWH1	Unknown function	0.46
C7_00420C_A	Unknown function	0.46
THI13	Thiamin pyrimidine synthase	0.46
C2_04330C_A	Putative oxidoreductase	0.46
ATP2	F1 beta subunit of F1F0 ATPase complex	0.46
PGA6	GPI-anchored cell wall adhesin-like protein	0.46
C1_00920W_A	Unknown function	0.46
CR_03530W_A	Mitochondrial inner membrane protein of unknown function	0.46
SEC22	Predicted role in ER to Golgi vesicle-mediated transport	0.46
C2_05550W_A	Predicted cytochrome b5-like heme/steroid binding domain	0.46
CR_07830C_A	Unknown function	0.47
C2_05130W_A	Unknown function	0.47
BUL4	Unknown function	0.47
C7_02140W_A	Unknown function	0.47
C1_04780C_A	Predicted role in negative regulation of TORC1 signaling	0.47
C7_00920C_A	Unknown function	0.47
CR_00310C_A	Unknown function	0.47
OPT7	Putative oligopeptide transporter	0.47
C4_02340W_A	Putative protease B inhibitor	0.47
C4_03860C_A	Unknown function	0.47
CR_08430W_A	Putative involvement in regulated synthesis of PtdIns(3,5)P(2)	0.47
CGR1	Negative regulator of yeast-form growth	0.47
C1_02220C_A	Unknown function	0.47
C5_03800W_A	Cytochrome c oxidase subunit	0.47
PLB4.5	Phospholipase B	0.47
CTM1	Putative cytochrome c lysine methyltransferase	0.47
RAM2	Subunit of heterodimeric protein geranylgeranyl transferase	0.47
NAG1	Glucosamine-6-phosphate deaminase	0.47
VPS33	Putative ATP binding and phosphatidylinositol binding activity	0.47
HHT21	Putative histone H3	0.47
CYP1	Peptidyl-prolylcis-trans isomerase	0.47
TLO5	Member of telomere-proximal genes; unknown function	0.47
VMA2	Vacuolar H(+)-ATPase	0.47
CR_00990W_A	Putative peptidase activity	0.47
TLO13	Member of telomere-proximal genes; unknown function	0.47
C1_10090C_A	Unknown function	0.47
C1_09310C_A	Unknown function	0.47
VPS16	Putative involvement in protein-vacuolar targeting	0.47
C3_04460W_A	Putative role in positive regulation of TORC1 signaling	0.47
CR_05320C_A	Unknown function	0.47
RIM20	Protein involved in the pH response pathway	0.47

RAD50	Putative DNA double-strand break repair factor	0.47
MDH1-3	Predicted malate dehydrogenase	0.47
PPS1	Putative dual specificity protein phosphatase	0.47
MTW1	Kinetochore component	0.47
RNR21	Ribonucleoside-diphosphate reductase	0.47
CTR1	Copper transporter	0.47
C6_03200W_A	Unknown function	0.48
C5_05350W_A	Predicted DNA primase activity	0.48
C5_02400W_A	Putative acyl-protein thioesterase	0.48
C7_02850W_A	Unknown function	0.48
CDC54	Putative pre-replication complex helicase subunit	0.48
C5_02320C_A	Putative role in GPI anchor biosynthetic process	0.48
C1_05920W_A	Unknown function	0.48
FAB1	Phosphatidylinositol 3-phosphate 5-kinase	0.48
LYS142	Zn(II)2Cys6 transcription factor	0.48
CR_08920W_A	Predicted oxidoreductase and dehydrogenase domains	0.48
CR_05040W_A	Unknown function	0.48
C1_09210C_A	Putative transporter	0.48
C2_09710C_A	Predicted role in actin cortical patch localization	0.48
C2_09980W_A	Protein with a PI31 proteasome regulator domain	0.48
CR_09230C_A	Putative role in re-entry into mitotic cell cycle	0.48
BUD7	Putative role in Golgi to plasma membrane transport	0.48
C6_00680C_A	Unknown function	0.48
C4_02330C_A	Unknown function	0.48
C4_03130W_A	Putative GPI transamidase component	0.48
COQ6	Predicted oxidoreductase activity	0.48
C7_03780C_A	Unknown function	0.48
C5_02050W_A	Predicted role in Golgi to endosome transport	0.48
C3_03380W_A	Unknown function	0.48
VMA13	Predicted proton-transporting ATPase	0.48
ATP14	Putative mitochondrial F1F0 ATP synthase subunit	0.48
MED11	Subunit of the RNA polymerase II mediator complex	0.48
FMO1	Putative oxidoreductase	0.48
GSG1	Putative subunit of the TRAPP complex	0.48
C3_06810W_A	Predicted ubiquitin-protein transferase activity	0.48
HCH1	Predicted regulator of heat shock protein Hsp90	0.48
COF1	Putative cofilin	0.48
HSP70	Putative hsp70 chaperone	0.48
PFK26	Putative 6-phosphofructo-2-kinase	0.48
C2_03210W_A	Unknown function	0.48
C5_03210C_A	Unknown function	0.48
PMT6	Protein mannosyl transferase	0.48
C2_07270W_A	Protein with an FMN-binding domain	0.48
ASH1	GATA-like transcription factor	0.48
SEC24	Protein with a possible role in ER to Golgi transport	0.48
C1_10230C_A	Unknown function	0.48
C1_11860W_A	Predicted essential RNA-binding G protein	0.48
C1_07690C_A	Putative nuclear export signal receptor activity	0.48
C2_10010C_A	Unknown function	0.49
NCR1	Putative vacuolar membrane protein	0.49
CR_10040W_A	Unknown function	0.49
OBPA	Putative oxysterol binding protein	0.49
ALD6	Putative aldehyde dehydrogenase	0.49
C5_03780C_A	Unknown function	0.49
SRV2	Adenylate cyclase-associated protein	0.49
ALG2	Putative mannosyl transferase	0.49
TRY4	C2H2 transcription factor	0.49
C5_02350C_A	Putative ubiquitin binding activity	0.49

C7_02920W_A	Unknown function	0.49
HSP78	Putative heat-shock protein	0.49
TFC4	Putative RNA polymerase III transcription initiation factor	0.49
PCL2	Cyclin homolog	0.49
C2_03800C_A	Putative role in Golgi vesicle transport	0.49
MDM34	Putative zinc finger transcription factor	0.49
C1_04700C_A	Unknown function	0.49
ECM33	GPI-anchored cell wall protein	0.49
ASH2	Putative histone methyltransferase activity	0.49
LSC1	Putative succinate-CoA ligase subunit	0.49
NIF3	Unknown function	0.49
CR_10020C_A	Predicted oligosaccharide binding activity	0.49
C1_09340C_A	Unknown function	0.49
SEC26	Secretory vesicles coatomer complex protein	0.49
MIM1	Predicted involvement in outer membrane protein import	0.49
MED20	Subunit of the RNA polymerase II mediator complex	0.49
VPS11	Protein involved in protein trafficking	0.49
C6_02190C_A	Putative serine/threonine/tyrosine (dual-specificity) kinase	0.50
C2_09860C_A	Putative monooxygenase	0.50
TAL1	Transaldolase	0.50
CR_07640C_A	Putative ATPase activator activity	0.50
BUB3	Predicted kinetochore checkpoint component	0.50
CRM1	Predicted involvement in protein nuclear export	0.50
YOX1	Putative homeodomain-containing transcription factor	0.50
C4_01430C_A	Unknown function	0.50
C7_01720W_A	Flavin-linked sulfhydryl oxidase	0.50
CR_03690W_A	Predicted hydrolase activity	0.50
C6_00660C_A	Putative Arp2/3 complex binding activity	0.50
C6_02560W_A	Putative oxidoreductase	0.50
GTT11	Glutathione S-transferase	0.50
VMA4	H+ transporting ATPase E chain	0.50
SMI1B	Putative cell wall assembly regulatory protein	0.50

Table AIII.5 List of progesterone-responsive genes in *Candida albicans* SC5314 biofilms grown 24h at pH 4. Were selected and listed here *C. albicans* SC5314 genes found to be up- and down-regulated in biofilms grown 24 h in RPMI at pH 4, in the presence (+ PRG) and/or absence (-PRG) of 2 μM of progesterone, and whose expression differed more than 40% between the two biofilm-forming conditions. Progesterone-responsive genes are presented in four groups *i)* genes up-regulated in biofilms formed in the presence or absence of progesterone, but showing a stronger induction in the presence of the hormone (42 genes); *ii)* genes more strongly up-regulated in biofilms without progesterone than on its presence (87 genes); *iii)* genes more strongly down-regulated in biofilms formed without progesterone (12 genes); *iv)* genes more strongly down-regulated in the progesterone-exposed biofilms (79 genes). Progesterone-decreased genes (groups *ii* and *iv*) required for biofilm formation (underlined) and/or analysed by qRT-PCR (Figure 5.7-B) are highlighted in bold. The biological function indicated is based on the information available at Candida Genome Database.

Group *i)*				
Gene	**Biological function**	**mRNA** + PRG	**mRNA** - PRG	**mRNA + PRG / mRNA - PRG**
ERG6	Protein involved in ergosterol biosynthesis	12.65	5.22	2.43
CDR2	Multidrug transporter of ABC superfamily	3.15	1.35	2.33
ICL1	Isocitrate lyase	2.15	0.97	2.23
PGA31	Cell wall protein involved in response to drug	10.18	4.85	2.10
PGA45	Putative GPI-anchored cell wall protein	20.17	9.66	2.09
CDR1	Multidrug transporter of ABC superfamily	3.15	1.53	2.06
POT1	Putative peroxisomal 3-oxoacyl CoA thiolase	2.61	1.30	2.00
C3_02870C_A	Unknown function	4.74	2.49	1.90
GAP2	General amino acid permease	2.14	1.18	1.82
ATO1	Putative transmembrane protein	7.62	4.31	1.77
C7_02260W_A	Unknown function	3.23	1.85	1.74
INO1	Inositol-1-phosphate synthase	47.93	28.05	1.71
FAS2	Alpha subunit of fatty-acid synthase	2.43	1.44	1.68
C2_05980C_A	Putative acyl-CoA hydrolase activity	3.17	1.89	1.68
PTH2	Putative cAMP-independent regulatory protein	3.60	2.19	1.64
AGP3	Putative serine transporter	5.12	3.20	1.60
C4_00250W_A	Unknown function	3.48	2.18	1.59
FAT1	Predicted enzyme of sphingolipid biosynthesis	2.12	1.33	1.59
GIT2	Putative glycerophospho inositol permease	5.04	3.19	1.58
CSA2	Extracellular protein involved in iron assimilation	4.07	2.58	1.58
REP1	Protein involved in regulation of MDR1	2.59	1.65	1.57
CDC13	Essential protein	2.48	1.59	1.56
C6_03600C_A	Putative cytochrome P450	2.22	1.43	1.55
CIS2	Putative role in regulation of cell wall	3.14	2.04	1.54
C3_05280C_A	Predicted fatty acid acyl transferase	2.40	1.57	1.53
C4_07020C_A	Unknown function	2.61	1.71	1.52
RHD3	GPI-anchored yeast-associated cell wall protein	2.83	1.89	1.50
AGP2	Amino acid permease	5.16	3.46	1.49
RBR2	Cell wall protein	2.12	1.42	1.49
SCW4	Putative cell wall protein	3.30	2.23	1.48
ANT1	Peroxisomal adenine nucleotide transporter	2.83	1.93	1.47
HGT1	High-affinity MFS glucose transporter	13.45	9.18	1.47
C7_02280W_A	Unknown function	4.54	3.11	1.46
HGT2	Putative MFS glucose transporter	15.45	10.57	1.46
ARO9	Aromatic transaminase	2.53	1.73	1.46
GPD2	Surface protein similar to dehydrogenase	3.10	2.14	1.45
C7_02010C_A	Predicted aldehyde dehydrogenase	2.40	1.66	1.44
FRP2	Putative ferric reductase	3.79	2.65	1.43
RHR2	Putative glycerol 3-phosphatase	2.33	1.64	1.43
FUN12	Translation initiation factor	2.22	1.56	1.42

CR_09920W_A	Predicted amino acid transport domain	5.23	3.67	1.42
DAP2	Putative dipeptidyl aminopeptidase	2.44	1.73	1.41

Group *ii)*

Gene	Biological function	mRNA + PRG	mRNA - PRG	mRNA + PRG / mRNA - PRG
COX3B	Cytochrome c oxidase, role in cellular respiration	1.54	10.97	0.14
NAD3	NADH dehydrogenase, role in cellular respiration	1.38	9.49	0.15
NAD2	NADH dehydrogenase, role in cellular respiration	1.19	7.81	0.15
ATP9	ATP synthase required for ATP synthesis	1.15	6.63	0.17
ATP6	ATP synthase required for ATP synthesis	1.52	7.84	0.19
NAD6	NADH dehydrogenase, role in cellular respiration	1.69	8.54	0.20
COX2	Cytochrome c oxidase, role in cellular respiration	1.76	8.56	0.21
NAD4	NADH dehydrogenase, role in cellular respiration	1.78	8.30	0.21
NAD5	NADH dehydrogenase, role in cellular respiration	1.85	8.59	0.21
NAD1	NADH dehydrogenase, role in cellular respiration	2.10	9.69	0.22
NAD4L	NADH dehydrogenase, role in cellular respiration	1.63	7.05	0.23
C3_00020W_A	Unknown function	1.61	6.14	0.26
CR_06350C_A	Unknown function	0.91	3.26	0.28
WOR3	Transcription factor, role in phenotypic switching	0.85	2.63	0.32
C3_00010C_A	Unknown function	1.53	4.10	0.37
C4_07260W_A	Unknown function	1.68	4.47	0.38
ATP8	ATP synthase required for ATP synthesis	1.15	3.03	0.38
C2_01760C_A	Unknown function	1.36	3.48	0.39
C2_01730W_A	Putative role in chromosome segregation	0.85	2.11	0.40
C4_02380W_A	Unknown function	0.90	2.06	0.43
C3_00040W_A	Unknown function	1.50	3.43	0.44
SUT1	Transcription factor involved in sterol uptake	0.96	2.16	0.44
BRG1	Transcription factor	0.89	2.01	0.44
C2_00750W_A	Plasma membrane-associated protein	1.40	2.97	0.47
RME1	Zinc finger protein; putative meiosis control	2.20	4.55	0.48
C3_00030C_A	Protein with a predicted helicase domain	1.73	3.54	0.49
C1_00270W_A	Predicted ORF in retrotransposon Tca8	1.17	2.39	0.49
MNN47	Unknown function	2.21	4.45	0.50
RPL33	Ribosomal 60S subunit protein	1.14	2.19	0.52
PRN3	Protein similar to pirin; unknown function	1.53	2.84	0.54
C6_03670C_A	Unknown function	2.15	3.97	0.54
PRN1	Protein similar to pirin; unknown function	1.61	2.90	0.56
TEC1	Transcription factor	3.20	5.64	0.57
C7_01510W_A	Predicted membrane transporter	1.24	2.16	0.57
PGA11	Putative GPI-anchored protein; unknown function	1.16	2.03	0.57
GPR1	Plasma membrane G-protein-coupled receptor	2.35	4.09	0.58
MRV4	Unknown function	1.39	2.42	0.58
CR_06650C_A	Unknown function	3.15	5.42	0.58
CHR1	Predicted ATP-dependent RNA helicase	1.61	2.71	0.60
HXT5	Putative sugar transporter	1.43	2.38	0.60
TPK1	cAMP-dependent protein kinase	2.32	3.86	0.60
C3_05450C_A	Unknown function	1.65	2.74	0.60
C2_05040C_A	Unknown function	1.23	2.05	0.60
C7_02630W_A	Unknown function	1.23	2.03	0.60
GIT1	Glycerophosphodiester transporter	1.30	2.14	0.60
C5_04910W_A	Protein required for maturation of 18S rRNA	1.88	3.08	0.61
TAR1	Putative regulator of cellular respiration	1.74	2.85	0.61
C1_01130W_A	Putative ubiquitin ligase complex component	1.99	3.24	0.61
WOR1	Transcription factor of phenotypic switching	1.32	2.16	0.61
C5_04980W_A	Putative adhesin-like protein	1.63	2.64	0.62
C1_01510W_A	Unknown function	4.67	7.57	0.62

MDR1	Plasma membrane multidrug efflux pump	2.57	4.14	0.62
IFE2	Putative alcohol dehydrogenase	1.50	2.41	0.62
CR_01160W_A	Unknown function	1.32	2.12	0.62
PGA37	Putative GPI-anchored protein	1.78	2.82	0.63
SFL2	Transcription factor required for filamentation	1.79	2.84	0.63
C5_00510W_A	Unknown function	1.29	2.04	0.63
FEN1	Putative fatty acid elongase	1.60	2.53	0.63
HAP2	Transcription factor, role in low-iron induction	1.37	2.16	0.63
MNN22	Alpha-1.2-mannosyltransferase	1.44	2.27	0.63
PBR1	Protein required for adhesion/ biofilm formation	2.51	3.92	0.64
SWE1	Putative protein kinase required for virulence	1.36	2.11	0.64
OPT9	Putative pseudogene similar to OPT1 transporter	1.62	2.49	0.65
CR_00460C_A	Predicted role in pre-18S rRNA processing	1.86	2.82	0.66
C2_04700C_A	U3-containing 90S preribosome complex protein	1.75	2.63	0.66
AHR1	Transcription factor involved in adhesion	1.76	2.62	0.67
CRL1	Predicted GTPase	1.56	2.33	0.67
SMM1	Putative dihydrouridine synthase	2.61	3.87	0.68
GPX2	Predicted glutathione peroxidase	1.59	2.36	0.68
NIP7	Putative nucleolar protein	1.88	2.76	0.68
RPC19	Putative RNA polymerases I and III subunit AC19	2.26	3.30	0.68
C6_01780C_A	Predicted chloride transporter	2.72	3.97	0.69
C2_10160W_A	Secreted protein	6.38	9.30	0.69
C1_11050W_A	Predicted nucleic acid binding protein	1.59	2.32	0.69
C3_06760W_A	Putative role in rRNA processing	1.92	2.78	0.69
HYR1	Hyphal cell wall protein involved in adhesion	6.82	9.87	0.69
QDR1	Putative antibiotic resistance transporter	3.96	5.71	0.69
C7_04080C_A	Putative adhesin-like protein	1.54	2.21	0.70
C1_12580W_A	Putative vacuole membrane localization	1.58	2.26	0.70
C2_05960C_A	Unknown function	1.44	2.07	0.70
C1_02450C_A	Predicted role in cellular bud site selection	2.46	3.52	0.70
C5_03740W_A	Unknown function	1.70	2.43	0.70
LAC1	Ceramide synthase	1.48	2.12	0.70
C4_01220C_A	Protein with a glycoside hydrolase domain	1.59	2.26	0.70
CFL2	Putative oxiredutase	1.41	2.01	0.70
PZF1	Transcription factor	2.21	3.13	0.70
IPK1	Putative role in organophosphate metabolism	1.83	2.59	0.71

Group *iii)*

Gene	Biological function	mRNA + PRG	mRNA - PRG	mRNA + PRG / mRNA - PRG
DUR1.2	Urea amidolyase	0.34	0.19	1.81
PXP2	Putative acyl-CoA oxidase	0.56	0.31	1.80
CR_05860W_A	Unknown function	0.80	0.49	1.63
CR_08920W_A	Predicted oxidoreductase and dehydrogenase	0.48	0.30	1.59
C5_02110W_A	Putative heat shock protein	0.41	0.26	1.57
C7_04280C_A	Putative catechol o-methyltransferase	0.70	0.47	1.50
MLS1	Malate synthase	0.52	0.35	1.48
C1_06860W_A	Predicted role in response to stress	0.62	0.43	1.46
TRY4	C2H2 transcription factor	0.49	0.33	1.46
C1_07080W_A	Suggested role in chromosome maintenance	0.61	0.42	1.46
HGT19	Putative MFS glucose/myo-inositol transporter	0.35	0.24	1.45
CIT1	Citrate synthase	0.54	0.38	1.45

Group *iv)*

Gene	Biological function	mRNA + PRG	mRNA - PRG	mRNA + PRG / mRNA - PRG
CBF1	Transcription factor	0.41	1.00	0.41
ARG3	Putative ornithine carbamoyltransferase	0.22	0.53	0.42

CR_09530C_A	Predicted endonuclease	0.30	0.72	0.42
CRZ2	Transcription factor	0.19	0.45	0.43
HSP21	Heat shock protein	0.21	0.45	0.46
HAK1	Putative potassium transporter	0.17	0.35	0.49
ZRT3	Unknown function	0.18	0.36	0.49
RSD1	Predicted short chain dehydrogenase	0.13	0.27	0.50
YKE2	Possible heterohexameric Gim/prefoldin protein	0.22	0.43	0.50
CIRT4B	Predicted involvement in DNA binding	0.28	0.54	0.52
C6_03370W_A	Unknown function	0.29	0.55	0.52
C1_07160C_A	Protein conserved among the CTG-clade	0.09	0.17	0.52
HWH1	Unknown function	0.46	0.86	0.54
PGK1	Phosphoglycerate kinase	0.28	0.52	0.54
BUB3	Predicted kinetochore checkpoint component	0.50	0.92	0.54
ZRT2	Zinc transporter	0.21	0.38	0.54
SNG4	Putative membrane transporter	0.45	0.83	0.55
OPT7	Putative oligopeptide transporter	0.47	0.85	0.55
C2_00760C_A	Unknown function	0.26	0.48	0.55
DEF1	RNA polymerase regulator; role in filamentation	0.37	0.67	0.56
C2_06600W_A	Predicted kinase	0.35	0.63	0.56
TIM22	Predicted role in protein import	0.44	0.78	0.56
CR_09070C_A	Unknown function	0.20	0.36	0.56
C2_08100W_A	Putative mitochondrial cell death effector	0.32	0.57	0.57
UCF1	Predicted involvement in generation of energy	0.12	0.21	0.57
CR_09580C_A	Putative involvement in proteasome assembly	0.33	0.58	0.57
RIM1	Putative single-stranded DNA-binding protein	0.35	0.61	0.58
C7_00350C_A	Unknown function	0.45	0.78	0.58
C6_03200W_A	Unknown function	0.48	0.82	0.58
HSP31	Putative heat shock protein	0.21	0.35	0.59
TPS3	Predicted trehalose-phosphate synthase	0.25	0.42	0.59
ARO10	Aromatic decarboxylase	0.18	0.31	0.59
CSR1	Transcription factor	0.36	0.60	0.59
HCH1	Predicted regulator of heat shock protein Hsp90	0.48	0.81	0.60
C4_00990W_A	Metal ion transporter domain-containing protein	0.45	0.76	0.60
CTM1	Putative cytochrome c lysine methyltransferase	0.47	0.77	0.61
OSM1	Putative flavoprotein unit of fumarate reductase	0.35	0.56	0.61
CR_06500C_A	Unknown function	0.32	0.52	0.62
C3_05840W_A	Predicted monooxygenase activity	0.38	0.62	0.62
NTF2	Putative nuclear envelope protein	0.38	0.61	0.63
MET28	Predicted transcription factor	0.44	0.71	0.63
C1_09210C_A	Putative transporter	0.48	0.76	0.63
GLK1	Putative glucokinase	0.24	0.39	0.63
C5_00400C_A	Unknown function	0.33	0.53	0.63
C5_00390C_A	Unknown function	0.33	0.53	0.64
C7_00920C_A	Unknown function	0.47	0.73	0.64
BUD7	Putative role in Golgi to membrane transport	0.48	0.74	0.64
HSP78	Putative heat-shock protein	0.49	0.76	0.65
DAG7	Secretory protein	0.41	0.62	0.65
C2_09980W_A	Protein with proteasome regulator domain	0.48	0.73	0.65
CCP1	Putative Cytochrome-c peroxidase	0.12	0.18	0.65
SNO1	Predicted role in pyridoxine metabolism	0.35	0.53	0.66
C1_12720C_A	Unknown function	0.27	0.40	0.66
CR_06510W_A	Unknown function	0.27	0.40	0.66
SNL1	Predicted role in protein synthesis	0.40	0.60	0.66
C5_02320C_A	Putative role in GPI anchor biosynthetic process	0.48	0.72	0.66
C7_00340C_A	Putative transporter activity	0.31	0.46	0.66
ENO1	Enolase involved in glycolysis	0.22	0.34	0.67
HSP104	Heat-shock protein	0.44	0.66	0.67
SPC3	Essential protein	0.34	0.50	0.67

NAG4	Putative transporter	0.40	0.59	0.68
DAK2	Putative dihydroxyacetone kinase	0.16	0.24	0.68
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	0.25	0.36	0.68
PGM2	Putative phosphoglucomutase	0.41	0.61	0.68
CDC55	Putative protein phosphatase activity	0.41	0.59	0.68
PCL1	Cyclin homolog	0.39	0.57	0.68
CDC37	Chaperone for Crk1p	0.43	0.63	0.68
TLO1	Member of a family of telomere-proximal genes	0.44	0.64	0.68
CR_00750C_A	Putative histone demethylase activity	0.45	0.66	0.68
C1_14090W_A	Putative role in protein assembly	0.19	0.28	0.68
PGI1	Predicted Glucose-6-phosphate isomerase	0.30	0.43	0.69
CFL5	Predicted Ferric reductase	0.31	0.45	0.69
GLK4	Putative glucokinase	0.26	0.37	0.69
CAS1	Putative transcription factor	0.29	0.42	0.69
C1_05920W_A	Unknown function	0.48	0.69	0.69
C2_00770W_A	Unknown function	0.31	0.44	0.70
MTW1	Kinetochore component	0.47	0.68	0.70
CR_00820C_A	Putative ribosome binding activity	0.30	0.44	0.70
TLO5	Telomere-proximal gene of unknown function	0.47	0.68	0.70