

**Prostaglandin E₂ production by *Candida albicans* and
*Candida dubliniensis***

by

Ruan Ells

**Submitted in accordance with the requirements for the degree
Philosophiae Doctor**

In the

**Department of Microbial, Biochemical and Food Biotechnology
Faculty of Natural and Agricultural Sciences
University of the Free State
Bloemfontein
South Africa**

Promotor: Dr. C.H. Pohl-Albertyn

Co-promotors: Prof. J.L.F. Kock

Prof. J. Albertyn

November 2011

This thesis is dedicated to the following people: My wife Léandri Ells; father, R. Ells; mother, L. Ells; sister, M. Ells and brother, J. Ells.

ACKNOWLEDGEMENTS

I wish to thank and acknowledge the following:

- **Dr. C. H. Pohl-Albertyn**, for all her guidance in making this project a success, her encouragement throughout the past three years, her confidence in me and being a good role model for me.
- **Prof. J. Albertyn**, for his assistance, guidance, advice in this project and always having an open door for me.
- **Prof. J. L. F. Kock**, for his assistance, guidance and advice in this project.
- **Prof. P. W. J. van Wyk** and **Dr. B. Janecke**, for assistance with the SEM.
- **Prof. D. Berger** and **Mr. N. Olivier** from the ACGT Microarray facility at the University of Pretoria for assistance with microarray analysis.
- **Dr. B. Visser** and his **Post graduate students** for assistance with RT-qPCR analysis.
- **The South African National Control Laboratory for Biological Products** at the UFS for supplying and guidance with the Hep2C epithelial cells.
- **Prof. A. Hugo** for assistance with GC analysis.
- **Dr. G. Kemp** for assistance with LCMS/MS analysis.
- **Prof. H. C. Swart & Dr. L. Coetsee** for assistance with NanoSAM analysis.
- **Mrs. A. van Wyk** for her support and providing the yeast cultures.
- **Mrs. E. van den Heever** for being patient in placing numerous orders and **Mrs. N. Agenbag** for support in the lab.
- **National Research Foundation**, South Africa, the **Blue Skies Programme** and the **UFS Advanced Biomolecular Research Cluster Programme** for the financial support.

Personal acknowledgements:

- My wife, **Mrs. L. Ells**, for all her love and support during the past three years.
- **My parents**, for giving me opportunities in life, their unconditional love and support.
- **Sister** and **brother** for their unconditional love and support.
- **My in-laws** for their acceptance and love.

- Friends, **Dr. C. W. Swart** and **Mr. F. C. Liebenberg** for being good friends and their support.
- **My fellow colleagues (Lab 28 & 49)**, for their assistance, support and encouragement during the past three years.
- **GOD Almighty**, for giving me strength and wisdom each day.

LIST OF ABBREVIATIONS USED

3-OH 8:0	3-OH caprylic acid
3-OH 9:1	3-OH nonaenoic acid
3-OH 10:0	3-OH capric acid
3-OH 14:2	3-OH tetradecadienoic acid
3-OH 14:3	3-OH tetradecatrienoic acid
3-OH 16:0	3-OH palmitic acid
3-OH 17:0	3-OH margaric acid
3-OH 18:0	3-OH stearic acid
3-OH 19:0	3-OH nonadecanoic acid,
3-OH 19:1	3-OH nonadecenoic acid
3-OH 20:0	3-OH arachidic acid
3-OH 22:0	3-OH behenic acid
4:0	butyric acid
7,8-diOH 18:2	7,8-dihydroxylinoleic acid
8-OH 18:2	8-hydroxylinoleic acid
14:0	myristic acid
16:1	palmitoleic acid (n-7)
16:2	palmitolinoleic acid (n-6)
18:1	oleic acid (n-9)
18:2	linoleic acid (n-6)
18:3	linolenic acid (n-3)
20:2	eicosadienoic acid (n-6)
22:4	adrenic acid (n-6)
AA	arachidonic acid [20:4(n-6)]
ABC	ATP Binding Cassette
ABT	1-aminobenzotriazole
ACP	acyl carrier protein
ALDp	adrenoleukodystrophy protein
ASA	acetylsalicylic acid, aspirin
ATM	ammonium tetrathiomolybdate
BSA	bovine serum albumin

cAMP	cyclic AMP
CGD	<i>Candida</i> Genome Database
COX	cyclooxygenases
CRTH2	chemoattractant receptor-homologous molecule expressed on Th2
CYP450	cytochrome P450
DGLA	dihomo- γ -linolenic acid [20:3(n-6)]
DHA	docosahexaenoic acid [22:6(n-3)]
DP	D prostanoid receptor
EETs	epoxyeicosatrienoic acids
EF3	elongation factor-3
ELISA	enzyme-linked immunosorbent assay
EP	E prostanoid receptor
EPA	eicosapentaenoic acid [20:5(n-3)]
FAMES	fatty acid methyl esters
FAS	fatty acid synthesis
FCS	foetal calf serum
FP	F prostanoid receptor
GC-MS	gas chromatography-mass spectrometry
GLA	γ -linolenic acid [18:3(n-6), (c6,9,12)]
GPCRs	G-protein-coupled receptors
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
HODE	hydroxyoctadecadienoic acid
HOTE	hydroxyoctadecatrienoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
HTDE	hydroxytetradecadienoic acid
IL	interleukin
INF- γ	gamma interferon
IP	prostacyclin receptor
LCMS/MS	liquid chromatography-tandem mass spectrometry
LDS	linoleate diol synthase
LPS	lipopolysaccharide

LOX	lipoxygenases
LTA ₄ H	leukotriene A ₄ hydrolase
LTB ₄	leukotriene B ₄
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MDR	multidrug resistance
MEM	minimum essential medium
MFS	major facilitator superfamily
<i>MTL</i>	mating-type like
MRM	multiple reaction monitoring
MRP	multidrug resistance-associated protein
NanoSAM	nano scanning auger microscopy
NDGA	nordihydroguaiaretic acid
NE	non-enzymatic
NMIFA	non-methylene interrupted fatty acid
NMR	nuclear magnetic resonance
NSAID	non-steroidal anti-inflammatory drug
OH	hydroxyl
OYE	old yellow enzyme
PBS	phosphate buffered saline
PDR	pleiotropic drug resistance
PEROX	peroxidase
PGD ₂	prostaglandin D ₂
PGE ₂	prostaglandin E ₂
PGF _{2α}	prostaglandin F _{2α}
PGHS	prostaglandin endoperoxide synthase
Pgp	P-glycoproteins
PLA ₂	phospholipase A ₂
PLB	phospholipase B
PMN	polymorphonuclear
Ppo	psi-producing oxygenases
PPOH	6-(2-propargyloxyphenyl)hexanoic acid
psi	precocious sexual inducers
PUFA	polyunsaturated fatty acid

QS	quorum sensing
RIA	radioimmuno assay
RLI	RNase L inhibitor
ROS	reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SA	sciadonic acid [20:3(n-6)]
SEM	scanning electron microscope
THETE	trihydroxy-eicosatetraenoic acid
Th1	T helper cell type 1
Th2	T helper cell type 2
TLC	thin layer chromatography
TNF α	tumor necrosis factor alpha
TP	thromboxane receptor
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide
YM	yeast malt extract

TABLE OF CONTENTS

Title page.....	i
Dedications.....	ii
Acknowledgements.....	iii
List of abbreviations used.....	v

CHAPTER 1

Oxylipins in yeasts and other fungi

1.1. Motivation.....	2
1.2. Introduction.....	4
1.3. Occurrence of oxylipins in fungi.....	5
1.4. Oxylipin production.....	17
1.4.1. Mammalian pathways.....	18
1.4.1.1. COX pathway.....	18
1.4.1.2. LOX pathway.....	18
1.4.1.3. Cytochrome P450 or epoxygenase pathway.....	19
1.4.1.4. Mitochondrial fatty acid synthesis.....	20
1.4.1.5. Transcellular pathways.....	20
1.4.1.6. Non-enzymatic pathways.....	22
1.4.2. Fungal pathways.....	22
1.4.2.1. Filamentous fungi.....	23
1.4.2.2. <i>Dipodascopsis uninucleata</i>	25
1.4.2.3. <i>Candida albicans</i>	26
1.4.2.4. <i>Cryptococcus neoformans</i>	27
1.4.2.5. <i>Paracoccidioides brasiliensis</i>	29
1.5. Biological activity of oxylipins.....	29
1.5.1. Prostanoids.....	30
1.5.2. LOX products.....	31
1.5.3. Cytochrome P450-derived eicosanoids.....	32

1.5.4. Non-enzymatically-derived eicosanoids.....	32
1.5.5. Fungal-derived oxylipins.....	33
1.6. Conclusions.....	41
1.7. References.....	42

CHAPTER 2

Prostaglandin production by Candida biofilms

2.1. Abstract.....	66
2.2. Introduction.....	67
2.3. Materials and methods.....	68
2.3.1. <i>Strains used.....</i>	68
2.3.2. <i>Biofilm formation.....</i>	68
2.3.3. <i>Determination of extracellular prostaglandin concentration by ELISA.....</i>	69
2.3.4. <i>Mass spectrometry.....</i>	69
2.3.5. <i>Germ tube assay.....</i>	70
2.3.6. <i>Determination of biomass and cell viability.....</i>	71
2.3.7. <i>Inhibition of extracellular PGE₂ production.....</i>	71
2.3.8. <i>Statistical analysis.....</i>	71
2.4. Results and discussion.....	72
2.4.1. <i>Prostaglandin production by Candida biofilms.....</i>	72
2.4.2. <i>Effect of fungal PGE₂ on germ tube formation by the Candida species.....</i>	75
2.4.3. <i>Effect of inhibitors on PGE₂ production by the Candida biofilms.....</i>	76
2.5. Conclusions.....	81
2.6. Acknowledgements	82
2.7. References.....	82

CHAPTER 3

Sciadonic acid modulates prostaglandin E_2 and cytokine production by epithelial cells during infection with *Candida albicans* and *Candida dubliniensis*

3.1. Abstract	87
3.2. Introduction	88
3.3. Materials and methods	90
3.3.1. <i>Host cells and culture conditions</i>	90
3.3.2. <i>Epithelial cell lipid analysis</i>	91
3.3.3. <i>Yeast strains used</i>	91
3.3.4. <i>Infection of Hep2C cells with <i>Candida</i> species</i>	92
3.3.5. <i>Scanning electron microscopy (SEM)</i>	92
3.3.6. <i>Nano Scanning Auger Microscopy (NanoSAM)</i>	93
3.3.7. <i>Determination of prostaglandin E_2 production by <i>Candida</i> biofilms</i>	93
3.3.8. <i>Determination of biomass and cell viability of <i>Candida</i> biofilms</i>	94
3.3.9. <i>Determination of prostaglandin E_2 production by epithelial cells in response to <i>Candida</i></i>	94
3.3.10. <i>Determination of cytokines produced by epithelial cells in response to <i>Candida</i></i>	94
3.3.11. <i>Statistical analysis</i>	95
3.3.12. <i>Ethics approval</i>	95
3.4. Results and discussion	96
3.4.1. <i>Sciadonic acid is incorporated into epithelial cell lipids</i>	96
3.4.2. <i>Microscopical analyses of epithelial cells during infection</i>	99
3.4.3. <i>Sciadonic acid modifies prostaglandin E_2 production by <i>C. albicans</i>, <i>C. dubliniensis</i> and infected epithelial cells</i>	104
3.4.4. <i>Incorporation of sciadonic acid influences cytokine production by Hep2C cells in response to <i>C. albicans</i> and <i>C. dubliniensis</i></i>	109
3.5. Conclusions	114
3.6. Acknowledgements	115

3.7. References.....	115
----------------------	-----

CHAPTER 4

Identification of *Candida albicans* genes differentially expressed in the presence of arachidonic and sciadonic acid

4.1. Abstract.....	127
4.2. Introduction.....	128
4.3. Materials and methods.....	129
4.3.1. Strains used.....	129
4.3.2. Biofilm formation.....	129
4.3.3. RNA extraction.....	130
4.3.4. cDNA synthesis for microarray analysis.....	131
4.3.5. cDNA dye-coupling for microarray analysis	132
4.3.6. Hybridization.....	133
4.3.7. cDNA synthesis for Reverse transcriptase-qPCR analysis.....	133
4.3.8. Reverse transcriptase-qPCR analysis.....	134
4.4. Results and discussion.....	136
4.4.1. Microarray analysis.....	136
4.4.1.1. Molecular function and biological processes of regulated genes... 138	
4.4.1.1.1. Oxidoreductases.....	142
4.4.1.1.2. Hydrolases.....	142
4.4.1.1.3. Transporters.....	143
4.4.1.1.4. Response to oxidative stress.....	146
4.4.1.1.5. Carbohydrate metabolic process.....	147
4.4.1.1.6. Other genes differentially expressed.....	149
4.4.1.2. Cellular component.....	151
4.4.1.2.1. Membranes.....	152
4.4.2. Reverse transcriptase-qPCR analysis.....	154
4.5. Conclusions.....	155

4.6. Acknowledgements..... 156

4.7. References..... 156

4.8. Supplementary table..... 167

GENERAL DISCUSSION..... 182

References..... 184

SUMMARY..... 187

Keywords..... 188

OPSOMMING..... 189

Sleutelwoorde..... 190

CHAPTER 1

Oxylipins in yeasts and other fungi

1.1. Motivation

Candida albicans and *C. dubliniensis* are closely related dimorphic pathogenic yeasts capable of forming biofilms (Ramage et al., 2001a, b; Sullivan et al., 1995). *Candida albicans* infections are associated with the release of the bioactive molecule, arachidonic acid (AA) [20:4(n-6)], from the infected host cell membrane (Brash, 2001; Deva et al., 2001). The released AA can be used as a carbon source by *C. albicans* and as a precursor for the synthesis of yeast eicosanoids such as prostaglandin E₂ (PGE₂) (Deva et al., 2000; Noverr et al., 2003). Eicosanoids are involved in *C. albicans* infection, affecting the host's immune responses, enhancing vascular permeability and facilitating the invasion of the host tissue/cells, as well as enhancing germ tube/biofilm formation (Deva et al., 2001; Noverr et al., 2001; Noverr & Huffnagle, 2004). The production of eicosanoids by *C. dubliniensis* has not been studied, although its close relationship to *C. albicans* (Sullivan et al., 1995) might point to similar ability to produce eicosanoids. Although this is an important area of research, not much is known about the mechanisms and metabolic pathways involved in the production of eicosanoids, such as PGE₂, as well as the functions of these compounds in the biology of fungi and yeasts, especially *C. albicans*.

The supplementation of mammalian cells with n-3 fatty acids has received much attention due to their beneficial health effects, providing immunomodulatory and anti-inflammatory activities (Bagga et al., 2003; Culp et al., 1979; Goodnight et al., 1982; Serhan et al., 2002). However, uncontrolled production of n-3 fatty acid metabolites could also be detrimental to the host and the high rates of inflammatory and autoimmune diseases are usually due to an imbalance between n-6 and n-3 PUFA intake. However, the anti-inflammatory effect of n-6 fatty acids has been limited to γ -linolenic acid (GLA) [18:3(n-6)]. This n-6 fatty acid is not a direct precursor for prostaglandin metabolism, but is converted to dihomo- γ -linolenic acid (DGLA), [20:3(n-6)], which is then converted to anti-inflammatory 1-series prostaglandins and thromboxane A₁ (Kapoor & Huang, 2006).

A non-methylene interrupted n-6 fatty acid, sciadonic acid (SA) [20:3(n-6)], competes with AA for incorporation into the phospholipids of mammalian cells, but cannot be directly metabolized to produce prostaglandins (Berger et al., 2002; Berger & Jomard, 2001; Tanaka et al., 2001). This characteristic provides these fatty

acids with potential anti-inflammatory properties (Berger & Jomard, 2001; German et al., 1995).

With this as background the aim of this study became:

- 1) To evaluate eicosanoid production from exogenous AA by *C. albicans* and *C. dubliniensis* biofilms (Chapter 2).
- 2) To evaluate the effect of SA on the ability of *C. albicans* and *C. dubliniensis* to produce PGE₂ and inflammation using Hep2C epithelial cells as an infection model (Chapter 3).
- 3) To identify possible *Candida* genes differentially expressed during growth in the presence of AA and SA (Chapter 4). This will be done by the use of microarray analysis and qPCR analysis.

1.2. Introduction

Fatty acids are the main components of lipids and play a key role as structural components of cellular membranes, affecting the physical state of the membranes, as storage lipids and as signalling molecules that impact the immune system in various ways (Van Bogaert et al., 2011). Oxylipins is the collective term for oxygenated polyunsaturated fatty acids (PUFAs) and metabolites, and includes the eicosanoids, which are an important group of oxygenated C20 PUFAs (Kock et al., 2003). These compounds include the epoxy derivatives, hepoxilins, hydro(pero)xy fatty acids, leukotrienes, lipoxins, prostacyclins, prostaglandins and thromboxanes (Smith, 1989; Zeldin, 2001). In mammalian cells they are mainly synthesized from dihomo- γ -linolenic acid (DGLA) [20:3(n-6)], arachidonic acid (AA) [20:4(n-6)] and eicosapentaenoic acid (EPA) [20:5(n-3)] (Smith, 1989) as well as from docosahexaenoic acid (DHA) [22:6(n-3)] (Serhan et al., 2004). They are synthesized through the actions of cyclooxygenases (COX) (Murakami & Kudo, 2004), lipoxygenases (LOX) (Henderson, 1994), cytochrome P450s (CYP450s) (Zeldin, 2001; Zhu et al., 1995), or non-enzymatic (NE) pathways (Buczynski et al., 2009) (Figure 1). However, in fungi the precursors for oxylipin production are usually oleic acid [18:1(n-9)], linoleic acid [18:2(n-6)] and linolenic acid [18:3(n-3)] (Tsitsigiannis & Keller, 2007). These precursors are mostly present in the phospholipids and triacylglycerides.

Oxylipins are found in almost every living organism i.e. in mammalian cells (Funk, 2001), in plants (Groenewald & van der Westhuizen, 1997) and in lower organisms including bacteria, yeast and filamentous fungi (Lamacka & Sajbidor, 1995). In all of the above, these compounds are known to be pharmacologically potent with important biological activities. Oxylipins, such as eicosanoids have tissue dependent functions, such as vasodilation, platelet aggregation and pain induction in biological systems (Holland et al., 1988). As pharmaceuticals they are commonly used as pyretic agents and abortives. Even though these compounds have vast applications and are in high demand, they are very expensive to chemically synthesize in large quantities (Dixon, 1991; Yilmaz, 2001).

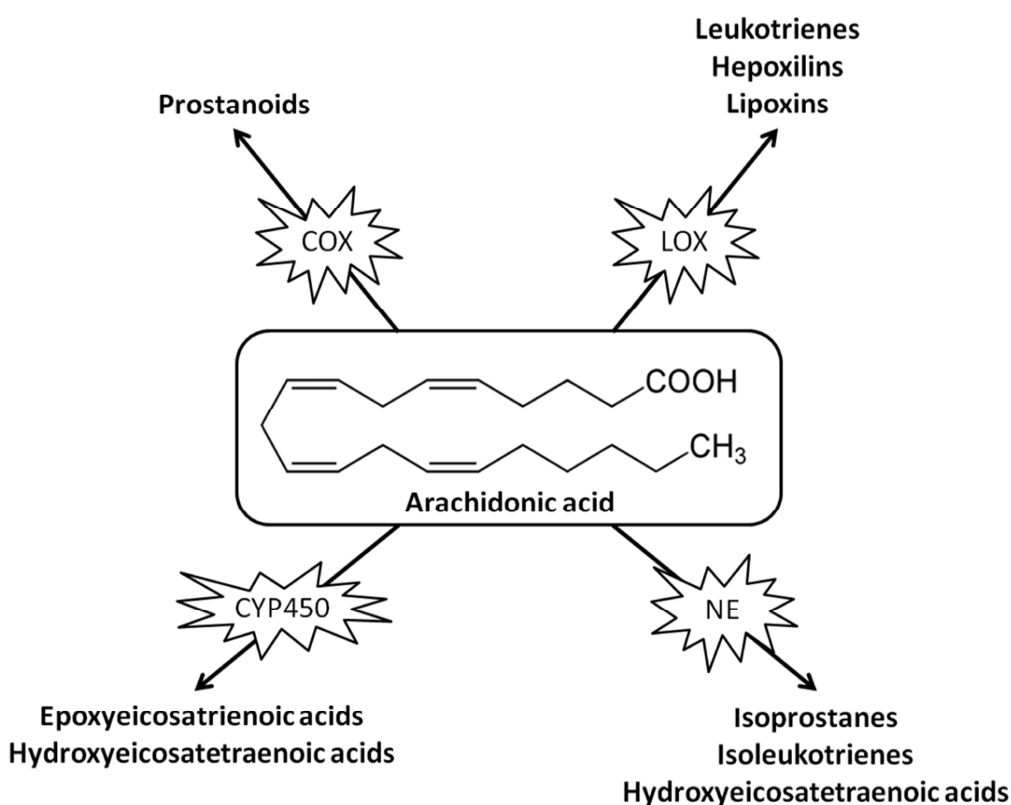


Figure 1. Schematic diagram indicating the biosynthesis of eicosanoids through COX, LOX, CYP450s and non-enzymatic (NE) pathways from arachidonic acid.

Most of what is known about oxylipins, such as eicosanoids, comes from the investigation of mammalian biology and very little is known about the biochemistry of eicosanoid production in the lower organisms, including fungi. This review will focus on the occurrence of oxylipins in fungi, including yeast, known metabolic pathways for the production of oxylipins as well as the possible roles and significance of these compounds in the biology of fungi.

1.3. Occurrence of oxylipins in fungi

As early as 1968, 3-hydroxy (OH) fatty acids were found to be produced by the then unidentified yeast strain NRRL Y-6954 (Vesonder et al., 1968). This compound was identified to be 3-OH palmitic acid (3-OH 16:0), and later found to be produced by the yeast, *Saccharomyces malanga* (Kurtzman et al., 1974). Later,

Sebolai and co-workers (2001) also demonstrated the production and possible function of 3-OH 16:0 by this yeast through gas chromatography-mass spectrometry (GC-MS) analysis and electron microscopy studies. In the closely related yeast, *Saccharomyces synnaedendra*, Sebolai and co-workers (2004) identified a novel cascade of saturated and unsaturated 3-OH oxylipins, using GC-MS. This included 3-OH 16:0, 3-OH margaric acid (3-OH 17:0), 3-OH stearic acid (3-OH 18:0), 3-OH 18:1, 3-OH nonadecanoic acid (3-OH 19:0), 3-OH nonadecenoic acid (3-OH 19:1), 3-OH arachidic acid (3-OH 20:0) and 3-OH behenic acid (3-OH 22:0).

In the late 1980s, a hormone-like compound derived from fatty acids, was found to induce the sexual cycle of the ascomycetous fungus *Aspergillus nidulans* (Champe et al., 1987; Champe & El-Zayat, 1989). This was later identified as precocious sexual inducers (psi), psiA α , psiA β and psiA γ , consisting out of a mixture of secreted hydroxylated 18:1(n-9), 18:2(n-6) and 18:3(n-3) fatty acids, respectively (Mazur et al., 1990). Mazur and co-workers (1991) also identified four other psi factors produced by *A. nidulans* as psiB α , psiB β , psiC α and psiC β . These psi compounds are characterized according to the position and number of the OH groups on the fatty acid backbone as psiA, psiB and psiC. They are also termed as psi β , psi α and psi γ according to the fatty acid from which they are derived i.e. 18:1(n-9), 18:2(n-6) and 18:3(n-3), respectively.

In environmental fungi, belonging to the Lipomycetaceae family (*Dipodascopsis*, *Lipomyces*, *Myxozyma*, *Waltomyces* and *Zygozoma*) and *Saccharomyces cerevisiae*, the production of prostaglandin F_{2 α} (PGF_{2 α}) was identified in cell extracts by radioimmuno assay (RIA) (Kock et al., 1991). The production of AA metabolites from exogenous AA by the Lipomycetaceae as well as the Dipodascaceae family (*Dipodascus*, *Galactomyces* and *Geotrichum*) was confirmed by the use of radiolabelled AA, thin layer chromatography (TLC) and autoradiography (Botha & Kock, 1993). Interestingly, only members of the Lipomycetaceae (*Dipodascopsis* and *Lipomyces anomalus*) produced acetylsalicylic acid (ASA) sensitive AA metabolites. These studies also emphasized the separation of the lipomycetaceous yeast, *Dipodascopsis*, from the Dipodascaceae family. In *Dipodascopsis uninucleata*, the possible production of prostaglandin E₂ (PGE₂) and/or prostaglandin D₂ (PGD₂), was indicated through blood platelet aggregation

studies (Kock et al., 1991). This research group also identified the production of an ASA sensitive prostacyclin by *D. uninucleata*, identified as α -pentanor PGF_{2 α} - γ -lactone, from exogenous AA, using GC-MS. More studies, using COX and LOX inhibitors, followed using the Lipomycetaceae family as a model and an AA cascade, speculated to be similar to that of mammalian cells, was identified (Kock et al., 1992). Similarly, in *D. tóthii*, two AA metabolites present during ascosporeogenesis, were identified as PGE₂ and PGF_{2 α} , using one dimensional TLC followed by scintillation counting (Botha et al., 1993).

In addition to prostaglandins, using various techniques including radio TLC, nuclear magnetic resonance (NMR) and GC-MS, it was found that *D. uninucleata* is capable of producing an ASA sensitive 3*R*-hydroxy 5,8,11,14-eicosatetraenoic acid (3*R*-HETE) from exogenous AA (van Dyk et al., 1991; Venter et al., 1997). Interestingly, the use of ASA to inhibit the production of prostaglandins and 3*R*-HETE in *D. uninucleata* resulted in the increased production of lipoxygenase products, i.e. 5-HETE, 12-HETE and 15-HETE (Coetzee et al., 1992). This suggested that both pathways exist in fungi and that fungi can shift between these pathways. Additionally, *D. uninucleata* is also capable of metabolizing various other fatty acids leading to the formation of 3-OH fatty acids (Venter et al., 1997). However, only 18:2(n-6), eicosatrienoic acid [20:3 (5,8,11)], 20:3 (11,14,17) and EPA were metabolized to its correspondent ASA sensitive 3-OH fatty acids. They were identified by GC-MS as 3-OH tetradecadienoic acid (3-OH 14:2), 3-OH 20:3, 3-OH tetradecatrienoic acid (3-OH 14:3) and 3-OH EPA, respectively. Fox and co-workers (2000a) also found the production of 3*R*-HETE from exogenous AA by cell-free enzyme extracts of this yeast, but it was not sensitive to ASA, but to antimycin A. This difference might be ascribed to the use of cell free extracts compared to whole cells in the previous study by Venter and co-workers (1997). This indicates that ASA may inhibit the uptake of AA into the cells, rather than the production of 3-HETE from AA. Fox and co-workers (2000a) also found that the addition of EPA lead to the production of 3-OH EPA, as previously indicated. However, no prostaglandin production was found.

Strauss and co-workers (2005) indicated the production of mainly ASA sensitive 3-OH caprylic acid (3-OH 8:0) as well as 3-OH capric acid (3-OH 10:0) *de*

novo and from exogenous AA by the brewers yeast *S. cerevisiae*, linking oxylipin production and flocculation. They also indicated the inability of these *S. cerevisiae* strains to produce 3*R*-HETE or inflammatory prostaglandins (e.g. PGF_{2α}), this is in contrast to previous findings (Kock et al., 1991), which were based on RIA.

Candida bombicola and *C. apicola* are known to produce sophorolipids, where the diglucoside, sophorose, is linked by a glycosidic bond through a hydroxyl group located at the terminal position of an OH-fatty acid (Prabhune et al., 2002). Through GC-MS analysis, it was identified that these yeasts produced sophorolipids consisting out of 19-HETE and 20-HETE when incubated in the presence of glucose and exogenous AA.

Brodowski and Oliw (1992) found the production of three major HETEs (i.e. 17-HETE, 18-HETE and 19-HETE) by the fungal parasite of wheat, *Gaeumannomyces graminis*, through the hydroxylation of exogenous AA whereas EPA was converted mainly to 17,18-diHETE. Similar results were obtained in 1969 in this yeast, then known as *Ophiobolus graminis* (Sih et al., 1969). In addition, this fungus also produced a variety of hydroxyoctadecadienoic acids (HODEs) and hydroxyoctadecatrienoic acids (HOTEs) from exogenous 18:2(n-6) and 18:3(n-3), respectively (Brodowsky et al., 1992). The closely related rice blast fungus, *Magnaporthe grisea*, was capable of metabolizing 18:2(n-6) to produce 8-hydroxylinoleic (8-OH 18:2) and 7,8-dihydroxylinoleic acid (7,8-diOH 18:2), where 8-OH 18:2 was further oxidized through a hydroxylation reaction to 8,16- and 8,17-diOH 18:2 (Cristea et al., 2003).

In addition, when the oomycetous fungus, *Leptomitus lacteus*, was incubated with exogenous AA, 17-HETE, 18-HETE and 19-HETE were produced (Akpınar et al., 1998). When this fungus was incubated with 18:2(n-6) a number of HODEs were also produced (Fox et al., 2000b). Interestingly, the oomycetous fungus, *Saprolegnia parasitica* converted AA through a LOX catalyzed reaction into 15*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HPETE) which was again isomerized into epoxy alcohols (Hamberg et al., 1986). This fungus also produced mainly n-6 PUFAs, including AA, when grown in semi-defined medium (Kendrick & Ratledge, 1992). *Saprolegnia diclina*, produced three trihydroxy-eicosatrienoic acids (i.e. 11,12,15-THETE, 11,14,15-THETE and 13,14,15-THETE) from exogenous AA

(Hamberg, 1986). It was also suggested that prostaglandin-like compounds are involved in the development of this fungus due to the dose dependent inhibition of growth by ASA and indomethacin (Herman & Herman, 1985). In the oomycetes, *Phytophthora nicotianae* and *P. citrophthora*, ASA inhibited growth as well as 3-OH oxylipin production, but it was found to be due to the inhibition of mitochondrial activity (Swart et al., 2011). This suggests that the observed reduction in growth in these oomycetes due to ASA, is not only due to the inhibition of oxylipins, but may also be due to an inhibition of the mitochondria.

Pohl and co-workers (1998) found that the filamentous soil fungus, *Mucor genevensis*, is capable of producing 3-hydroxy-5Z,8Z-tetradecadienoic acid (3-HTDE) from exogenous AA through a possible retroconversion of AA to 18:2(n-6). These 3-OH fatty acids were mainly found in the columellae, sporangia and aggregating sporangiospores. Interestingly, this genus has the ability to produce the eicosanoid precursors, AA, DGLA and EPA, similar to the closely related filamentous fungi *Mortierella alpina* and *M. isabelina* (Botha et al., 1997; Higashiyama et al., 2002; Kendrick & Ratledge, 1992; Lamacka & Sajbidor, 1998). These *Mortierella* species also produce the eicosanoids PGE₂ and PGF_{2α}, both extra- and intracellularly (Lamacka & Sajbidor, 1998) as well as HETEs (Akpinar et al., 1998).

There has been many reports of 3-OH oxylipin production by environmental fungi including *Ascoidea africana* (Bareetseng et al., 2005), *Eremothecium* spp. (Bareetseng et al., 2004; Kock et al., 2004, Leeuw et al., 2006, 2007), *Lipomyces* spp. (Smith et al., 2000), *Nadsonia* spp. (Bareetseng et al., 2004), *Saccharomycopsis* spp. (Sebolai et al., 2001, 2005), *Saturnispora saitoi* (Bareetseng et al., 2006) and *Schizosaccharomyces pombe* (Strauss et al., 2006). Eicosanoids can also be implicated in the pathogenesis of certain microorganisms. The production of eicosanoids in various pathogenic fungi was also indicated. The use of enzyme-linked immunosorbent assay (ELISA) identified the production of prostaglandins (PGD₂, PGE₂, PGF_{2α}) and leukotrienes [cysteinyl leukotrienes, leukotriene B₄ (LTB₄)] *de novo* as well as from exogenous AA in fungi including dermatophytes, subcutaneous pathogens as well as systemic pathogens belonging to species in the genera *Absidia*, *Aspergillus*, *Blastomyces*, *Epidermophyton*,

Fusarium, *Histoplasma*, *Microsporium*, *Penicillium*, *Rhizopus*, *Rhizomucor*, *Sporothrix* and *Trichophyton* (Noverr et al., 2002).

The production of a 3-OH fatty acid from exogenous AA was found in the pathogenic yeast, *Candida albicans* (Deva et al., 2000, 2001). This compound was identified by GC-MS as 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18 di-HETE) and was associated with the hyphal forms, possibly playing a role in morphogenesis and pathogenicity. In biofilms of the closely related yeast, *C. dubliniensis*, the production of 3,18 di-HETE from exogenous AA was also found (Ells, 2008). Similar oxygenated lipids, 1-hydroxy-3,7,11-trimethyl-2,6,10-dodecatriene, or farnesol, and 3(*R*)-HTDE are also produced by *C. albicans* (Nickerson et al., 2006; Nigam et al., 2011; Oh et al., 2001). These oxylipins regulate mycelial growth and have quorum sensing (QS) activity. In addition, Sebolai and co-workers (2007) indicated the presence of 3-OH nonaenoic acid (3-OH 9:1) accumulating in the capsule of *Cryptococcus neoformans* var. *neoformans* vegetative cells.

Noverr and co-workers (2001) indicated by the use of ELISA assays that the pathogenic yeasts, *C. albicans* and *Crypt. neoformans*, have the ability to produce and secrete prostaglandins *de novo* and that the addition of exogenous AA increased this production significantly. They referred to it as PGE_x due to the cross-reactivity observed with prostaglandins of the E class using prostaglandin immunoassays. Later, using mass spectrometry, it was verified as PGE₂ (Erb-Downward & Huffnagle, 2007; Erb-Downward & Noverr, 2007). *Candida albicans* and *Crypt. neoformans* can also produce other prostaglandins. This was indicated by using ELISA assays to identify the production of PGD₂ and PGF_{2α} as well as leukotrienes (cysteinyl leukotrienes, LTB₄) from exogenous AA (Noverr et al., 2002). Similar results were obtained by Erb-Downward and co-workers (2008) in *Crypt. neoformans*, however lysates from this yeast produced more PGF_{2α} compared to PGE₂, in contrast to *C. albicans*, where PGE₂ was the main prostaglandin produced. This eicosanoid production was found for planktonic cells, however the production of PGE₂, sensitive to COX inhibitors, *de novo* by *C. albicans* biofilms has also been reported (Alem & Douglas, 2004, 2005). The COX inhibitors used in the latter study also inhibited biofilm formation. Interestingly, the addition of PGE₂ together with ASA

completely removed biofilm inhibition by ASA. The authors concluded that biofilm development, morphogenesis and regulation of physiological processes in this yeast are regulated by COX-dependent synthesis of fungal prostaglandins.

It was also found that *C. albicans* is capable of metabolizing DHA and EPA, producing a range of oxygenated lipids, determined by liquid chromatography-tandem mass spectrometry (LCMS/MS) (Haas-Stapleton et al., 2007). Interestingly, resolvin E1 (RvE1) as well as its precursors, 5-hydroxyeicosapentaenoic acid (5-HEPE), 15-HEPE and 18-HEPE, similar to those produced by mammalian cells, were also found amongst these oxygenated lipids produced by *C. albicans* from EPA.

During the last few years there has also been an increase in other non-*albicans Candida* species as opportunistic human pathogens (Segal, 2005). These include *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. Recently, it was indicated, by analyzing the culture supernatants using an ELISA assay, that *C. glabrata* and *C. tropicalis* are capable of producing PGE₂ (Shiraki et al., 2008). *Candida albicans* and *C. tropicalis* produced considerable amounts of PGE₂, whereas *C. glabrata* produced only trace amounts. Interestingly, in the presence of human keratinocytes, important in cutaneous immune responses, *C. albicans*, *C. tropicalis* as well as *C. glabrata* produced 10-fold more PGE₂ with the keratinocytes alone producing only trace amounts of PGE₂. This indicates the involvement of PGE₂ during host pathogen interactions, specifically during superficial infections.

Tsitsigiannis and co-workers (2005a) used GC-MS to confirm the production of 18:1(n-9) and 18:2(n-6) derived oxylipins, psiBβ [8-hydroxy-9(Z)-octadecanoic acid] and psiBα [8-hydroxy-9(Z),12(Z)-octadecadienoic acid] respectively, by *ppo* enzymes in *A. nidulans*, similar to the results by Mazur and co-workers (1990, 1991). Additionally, the production of prostaglandins by *A. nidulans* and *A. fumigatus* from exogenous AA was also found using an ELISA assay (Tsitsigiannis et al., 2005b). However, due to cross reactivity of prostaglandins using this assay, the exact prostaglandins could not be identified. The use of a gene-silencing approach indicated that the same *ppo* enzymes were involved in prostaglandin production and also play an important part in virulence of *Aspergillus*. Recently, a new oxylipin, (8E,12Z)-10,11-dihydroxyoctadeca-8,12-dienoic acid, was identified in the plant

pathogen, *A. flavus*, (Qiao et al., 2011). Another plant pathogen, *Lasiodiplodia theobromae*, was found to produce (1*R*,2*R*)-3-Oxo-2-(2*Z*)-2-pentenyl-cyclopentaneacetic acid (jasmonic acid) from 18:3(n-3), via a pathway similar to the octadecanoid pathway in plants (Tsukada et al., 2010).

The pathogenic dimorphic fungus, *Paracoccidioides brasiliensis*, also produced prostaglandins from endogenous and exogenous AA (Biondo et al., 2010; Bordon et al., 2007). This was shown by measuring prostaglandin production with an ELISA assay. They referred to it as PGE_x due to the cross-reactivity observed with prostaglandins of the E class. The use of indomethacin and piroxicam not only decreased PGE_x production but also viability of the fungus.

The dimorphic lipophilic yeast, *Malassezia furfur*, was found to release AA from epithelial cells by phospholipase activity, which enhanced inflammatory responses during skin infections (Plotkin et al., 1998). This was confirmed through the use of high performance liquid chromatography (HPLC) and TLC analysis. Although not identified, the increase in inflammatory responses might be due to the production of prostaglandins by this yeast from the released AA. It was also indicated that *M. furfur* can oxidize 18:2(n-6) into hydroperoxides contributing to depigmentation of the skin (De Luca et al., 1996; Nazzaro-Porro et al., 1986).

A summary of the oxylipins produced by fungi is provided in Table 1.

Table 1: Summary of the discovery of oxylipins produced from polyunsaturated fatty acids by fungi.

Species	Eicosanoid	References
<i>Absidia corymbifera</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Ascoidea Africana</i>	3-OH fatty acids	Baretseng et al., 2005
<i>Aspergillus flavus</i>	(8 <i>E</i> ,12 <i>Z</i>)-10,11-dihydroxyoctadeca-8,12-dienoic acid	Qiao et al., 2011
<i>Aspergillus fumigatus</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α} ; prostaglandins	Noverr et al., 2002; Tsitsigiannis et al., 2005a
<i>Aspergillus nidulans</i>	hydroxylated C18 fatty acids (psi factors); prostaglandins	Mazur et al., 1990, 1991; Tsitsigiannis et al., 2005a, b
<i>Blastomyces dermatitidis</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Candida albicans</i>	1-OH-3,7,11-trimethyl-2,6,10-dodecatriene; 3(<i>R</i>)-HTDE; 3-OH-PGE ₂ ; 3,18 di-HETE; cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Alem & Douglas, 2004, 2005; Ciccoli et al., 2005; Deva et al., 2000, 2001; Ells, 2008; Erb-Downward & Huffnagle, 2007; Erb-Downward & Noverr, 2007; Nickerson et al., 2006; Nigam et al., 2011; Noverr et al., 2001, 2002; Oh et al., 2001; Shiraki et al., 2008

<i>Candida apicola</i>	19-HETE; 20-HETE	Prabhune et al., 2002
<i>Candida bombicola</i>	19-HETE; 20-HETE	Prabhune et al., 2002
<i>Candida dubliniensis</i>	3,18 di-HETE	Ells, 2008
<i>Candida glabrata</i>	PGE ₂	Shiraki et al., 2008
<i>Candida tropicalis</i>	PGE ₂	Shiraki et al., 2008
<i>Cryptococcus neoformans</i>	3-OH 9:1; cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Erb-Downward & Huffnagle, 2007; Erb-Downward & Noverr, 2007; Noverr et al., 2001, 2002; Sebolai et al., 2007
<i>Dipodascopsis tothii</i>	3R-HETE; ASA sensitive AA metabolite; PGE ₂ ; PGF _{2α}	Botha et al., 1993; Kock et al., 1991, 1992, 1997
<i>Dipodascopsis uninucleata</i>	3-OH 14:2; 3-OH 14:3; 3-OH 20:3; 3-OH 20:5; 3R-HETE; 5-HETE; 12-HETE; 15-HETE; ASA sensitive AA metabolite; PGD ₂ ; PGE ₂ ; PGF _{2α} ; PGF _{2α} -γ-lactone	Botha & Kock, 1993; Coetzee et al., 1992; Fox et al., 2000a; Kock et al., 1991, 1992; van Dyk et al., 1991; Venter et al., 1997
<i>Dipodascus</i> species	AA metabolite	Botha & Kock, 1993
<i>Epidermophyton floccosum</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Eremothecium</i> species	3-OH fatty acids	Bareetseng et al., 2004; Kock et al., 2004; Leeuw et al., 2006, 2007

<i>Fusarium dimerum</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Gaeumannomyces graminis</i>	17-HETE; 17,18-diHETE; 18-HETE; 19-HETE; HODEs; HOTEs	Brodowsky et al., 1992; Brodowski & Oliw, 1992; Sih et al., 1969
<i>Galactomyces geotrichum</i>	AA metabolite	Botha & Kock, 1993
<i>Geotrichum</i> species	AA metabolite	Botha & Kock, 1993
<i>Histoplasma capsulatum</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Leptomitus lacteus</i>	17-HETE; 18-HETE; 19- HETE; HODEs	Akpinar et al., 1998; Fox et al., 2000b
<i>Lipomyces</i> species	3-OH fatty acids; ASA sensitive AA metabolite; PGF _{2α}	Kock et al., 1991, 1992; Smith et al., 2000
<i>Magnaporthe grisea</i>	7,8-diOH 18:2; 8-OH 18:2; 8,16-diOH 18:2; 8,17- diOH 18:2	Cristea et al., 2003
<i>Malassezia furfur</i> (<i>Pityrosporum orbiculare</i>)	Hydroperoxy fatty acids	De Luca et al., 1996; Nazzaro-Porro et al., 1986
<i>Microsporum audouinii</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Microsporum canis</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Mortierella alpina</i>	HETE; PGE ₂ ; PGF _{2α}	Akpinar et al., 1998; Lamacka & Sajbidor, 1998

<i>Mortierella isabelina</i>	HETEs; PGE ₂ ; PGF _{2α}	Akpinar et al., 1998; Lamacka & Sajbidor, 1998
<i>Mucor genevensis</i>	3-HTDE	Pohl et al., 1998
<i>Myxozyma</i> species	PGF _{2α}	Kock et al., 1991
<i>Nadsonia</i> species	3-OH fatty acids	Bareetseng et al., 2004
<i>Paracoccidioides brasiliensis</i>	PGE _x	Biondo et al., 2010; Bordon et al., 2007
<i>Penicillium</i> species	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Phytophthora citrophthora</i>	3-OH fatty acids	Swart et al., 2010
<i>Phytophthora nicotianae</i>	3-OH fatty acids	Swart et al., 2010
<i>Rhizomucor pusillus</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Rhizopus</i> species	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Saccharomyces cerevisiae</i>	3-OH 8:0; 3-OH 10:0; PGF _{2α}	Kock et al., 1991; Strauss et al., 2005
<i>Saccharomycopsis malanga</i>	3-OH 16:0; 3-OH fatty acids	Kurtzman et al., 1974; Sebolai et al., 2001, 2005
<i>Saccharomycopsis synnaedendra</i>	3-OH 16:0; 3-OH 17:0; 3- OH 18:0; 3-OH 18:1; 3-OH 19:0; 3-OH 19:1; 3-OH 20:0; 3-OH 22:0	Sebolai et al., 2004

<i>Saprolegnia diclina</i>	11,12,15-THETE; 11,14,15-THETE; 13,14,15-THETE	Hamberg, 1986
<i>Saprolegnia parasitica</i>	15-HPETE	Hamberg et al., 1986
<i>Saturnispora saitoi</i>	3-OH fatty acids	Bareetseng et al., 2006
<i>Schizosaccharomyces pombe</i>	3-OH fatty acids	Strauss et al., 2006
<i>Sporothrix schenckii</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Trichophyton rubrum</i>	cysteinyl leukotrienes; LTB ₄ ; PGD ₂ ; PGE ₂ ; PGF _{2α}	Noverr et al., 2002
<i>Waltomyces lipofer</i>	AA metabolite	Botha & Kock, 1993
<i>Zygozoma</i> species	PGF _{2α}	Kock et al., 1991

1.4. Oxylipin production

The biosynthetic pathway for eicosanoid production in mammalian cells has been well studied and is used as a model to try and identify enzymes involved in this pathway in lower organisms, including fungi. The specific AA metabolites produced *in vivo* in mammalian cells are dependent upon the most active enzymes in specific tissues (Konturek & Pawlik, 1986). Cyclooxygenases, LOX, CYP450s and β -oxidation enzymes are known to add hydroxyl groups to AA (Yilmaz, 2001).

1.4.1. Mammalian pathways

Arachidonic acid is present in cell membranes (Yilmaz, 2001) and is released from the *sn*-2 position of phospholipids by a cytosolic phospholipase A₂ (PLA₂), after different types of stimulation such as chemical (antigens, autocoids, growth factors, hormones) or physical stimuli (electrical, stretching, squeezing, vibration) (Lambert, 1994; Newton & Roberts, 1997). This released AA (or exogenous AA) can be either re-acylated into the cell membrane or can be used to produce eicosanoids via different pathways (Figure 2) (Lambert, 1994; Yilmaz, 2001).

1.4.1.1. COX pathway

Cyclooxygenases, also known as prostaglandin endoperoxide synthases or prostaglandin H synthases, contain haem-iron, which catalyze the introduction of two oxygen molecules into AA (Figure 2) (Needleman et al., 1986; Smith et al., 1996). In mammalian cells, two isoforms of COX exist, COX-1 and COX-2, which are highly similar in structure and enzymatic activity, but differ in genetic regulation and biological roles. Cyclooxygenases perform two sequential reactions. Firstly AA is oxidized, during what is commonly referred to as the COX reaction, to an unstable endoperoxide, PGG₂, by a prostaglandin endoperoxide synthase (PGHS) (Lambert, 1994; Yilmaz, 2001). This is then followed by the reduction of PGG₂ to another unstable compound, endoperoxide PGH₂, through the peroxidase reaction (PEROX) of the enzyme. The latter compound can then be enzymatically metabolized by other enzymes including isomerases, reductases or synthases to biologically active prostanoids including the prostaglandins (PGD₂, PGE₂, PGF_{2α}, PGI₂) and thromboxanes (TXA₂, TXB₂) depending on the site of synthesis and the stimulus (Sigal, 1991; Yilmaz, 2001).

1.4.1.2. LOX pathway

Arachidonic acid can also be used as a substrate for several LOX enzymes, which are non-haem dioxygenases that catalyze the addition of one oxygen

molecule into AA at different positions (Figure 2). The hydroxylation and epoxygenation reactions lead to the synthesis of leukotrienes (e.g. LTB_4 , LTE_4), mono-, di-, and trihydro(pero)xy fatty acids (e.g. 5-HPETE, 5-HETE, 12-HETE, 15-HETE) as well as lipoxins (Lambert, 1994; Needleman et al., 1986; Samuelsson et al., 1987).

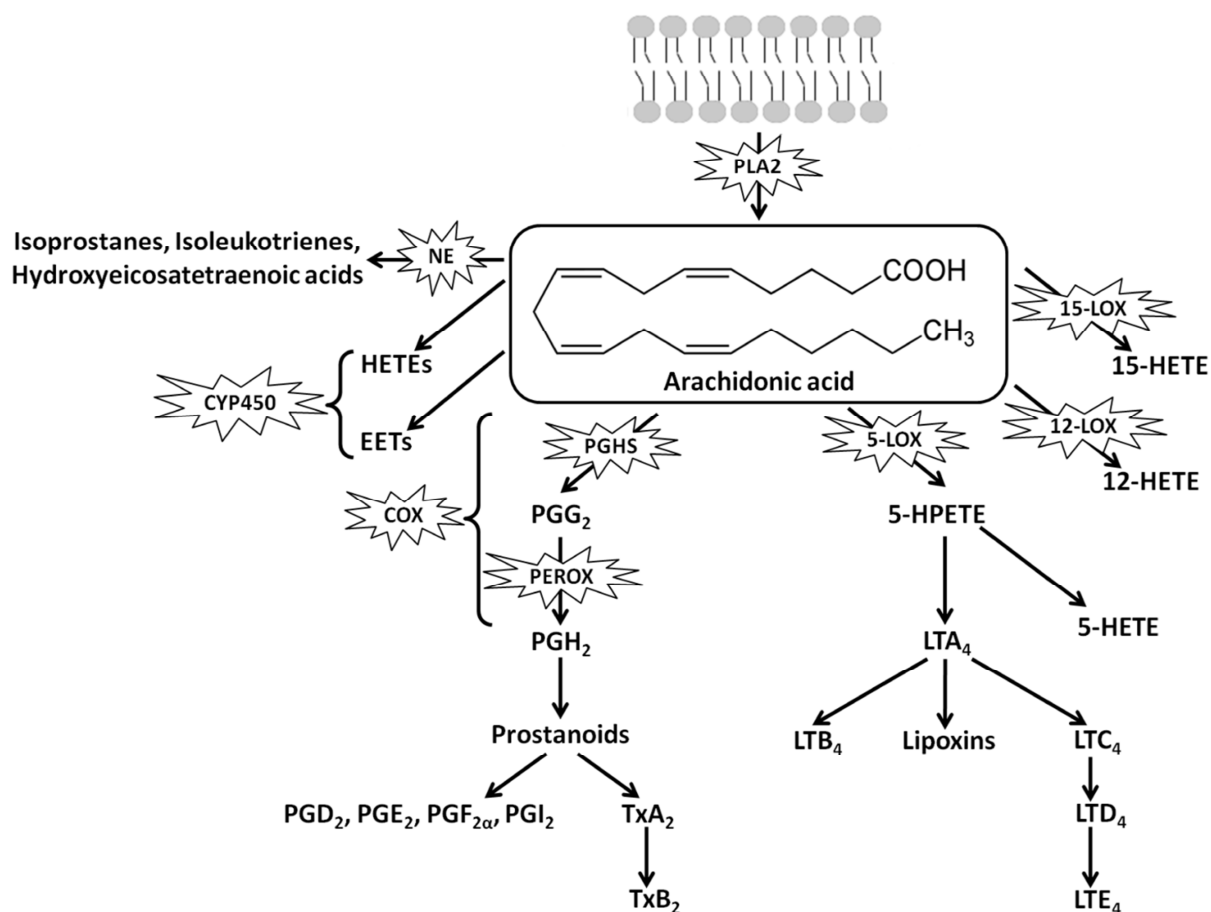


Figure 2. A schematic diagram of the biosynthetic pathway for eicosanoid production from arachidonic acid indicating the most important enzymes as well as possible eicosanoids produced.

1.4.1.3. Cytochrome P450 or epoxygenase pathway

Another important pathway similar to LOX, resulting in the hydroxylation and epoxygenation of AA, is the CYP450 or epoxygenase pathway. Cytochrome P450 represents the group of monooxygenases containing unique active haem proteins,

which form carbon monoxide complexes with a major absorption band at a wavelength of 450 nm. These enzymes are widely distributed among living organisms, including animals, plants and microorganisms (Lambert, 1994; Needleman et al., 1986) and can metabolize AA to epoxyeicosatrienoic acids (EETs) (epoxidation) and HETEs (ω -hydroxylase) by an NADPH-dependant mechanism (Figure 2) (Capdevila et al., 2000; Zeldin, 2001).

1.4.1.4. Mitochondrial fatty acid synthesis

Hydroxylated fatty acids can also be produced through mitochondrial fatty acid synthesis (FAS) type II (Ciccoli et al., 2005; Hiltunen et al., 2005). In eukaryotes, *de novo* fatty acid synthesis may occur in the cytoplasm, known as the cytosolic FAS type I pathway, and in the mitochondria via FAS type II (Hiltunen et al., 2009). The synthesis of fatty acids in eukaryotic mitochondria is highly conserved, uses a discrete set of enzymes and proceeds through a malonyl-CoA/acyl carrier protein (ACP)-dependent manner (Hiltunen et al., 2010). This pathway is known for the production of mainly short chain fatty acids i.e. 8:0, the substrate for mitochondrial lipoic acid synthesis ($C_8H_{14}O_2S_2$). However, the FAS II pathway also synthesizes longer fatty acids of up to 14-carbons in length from 2-carbon precursors (Hiltunen et al., 2010; Witkowski et al., 2007). This was found in mitochondrial matrix preparations from bovine heart. This pathway also produced 3-hydroxymyristoyl-ACP in bovine heart mitochondria (Carroll et al., 2003).

1.4.1.5. Transcellular pathways

Another group of eicosanoids that play a role in resolution of inflammation has recently been identified as the resolvins and protectins (Serhan et al., 2002, 2004). The production of these compounds involves the COX, LOX and CYP450 enzymes in a transcellular pathway (Figure 3) (Serhan et al., 2002, 2008a). These compounds are produced from the important n-3 PUFAs, DHA and EPA. They are referred to as resolvins of the E series if they are derived from EPA, while those synthesized from DHA either resolvins or protectins of the D series (Serhan et al., 2004). Resolvin E1

is produced when the non-steroidal anti-inflammatory drug (NSAID), ASA, acetylates COX-2 in vascular endothelial cells. This results in COX-2's catalytic activity being directed away from producing pro-inflammatory eicosanoids (i.e. prostaglandins and thromboxanes) from AA, and generates 18*R*-HEPE or 15*R*-HEPE from EPA. This is then further reduced to alcohols and epoxide intermediates via 5-LOX in polymorphonuclear (PMN) leukocytes and additional enzymes to form RvE1 and 15-epi-lipoxin-A₅. In addition, microbial as well as mammalian cells contain CYP450 that can also convert EPA to 18*R*-HEPE, in the absence of ASA, which is further oxygenated by 5-LOX (Serhan et al., 2008a). This suggests that RvE1 can also be formed during multi-species interactions, such as during inflammation as a result of a microbial infection. Additionally, the acetylation of COX-2 prevents the formation of prostaglandins, but the enzyme remains active to produce 15*R*-HETE which can then be metabolized by 5-LOX to produce anti-inflammatory ASA-triggered analogues of lipoxins known as 15 epimer lipoxins (Rajakariar et al., 2006; Serhan et al., 2008a).

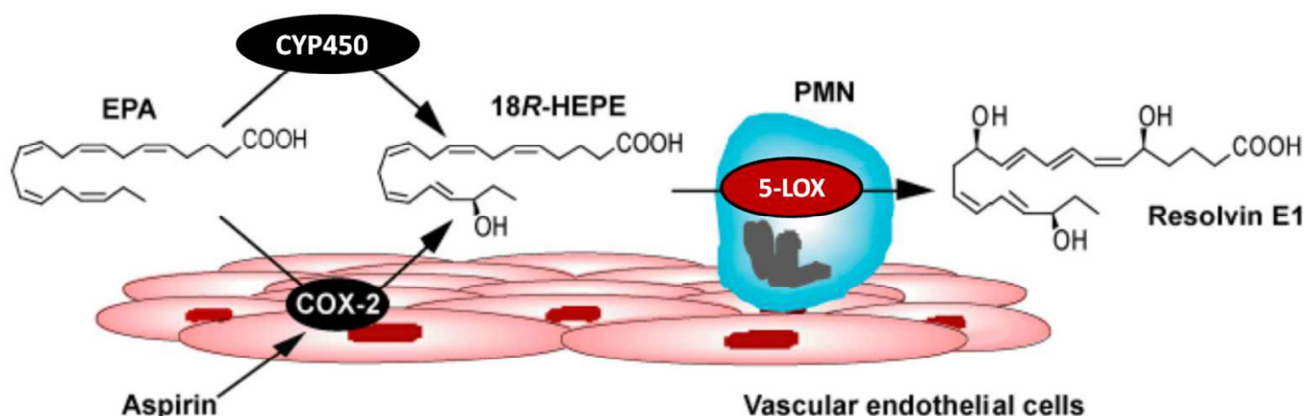


Figure 3. Transcellular biosynthetic pathway of resolvin E1 (RvE1) from eicosapentaenoic acid (EPA) through the acetylation of cyclooxygenase-2 (COX-2) as well as the aspirin (ASA) independent pathway. Cyclooxygenase-2 gets acetylated in the vascular endothelial cells by ASA and leads to the production of 18*R*-hydroxyeicosapentaenoic acid (18*R*-HEPE). This is then followed by the conversion to RvE1 by polymorphonuclear (PMN) leukocyte 5-lipoxygenase (5-LOX). Mammalian as well as microbial cytochrome 450 (CYP450) can convert EPA to 18*R*-HEPE. This intermediate can then be further converted via 5-LOX in PMN leukocytes and additional enzymes to form RvE1 (Arita et al., 2005).

1.4.1.6. Non-enzymatic pathways

Oxidative stress leads to the formation of free radicals contributing to a number of neurodegenerative and inflammatory diseases, cancer and atherosclerosis by causing damage to DNA, proteins and lipids (Milne et al., 2008). Non-enzymatic reactions can also produce prostaglandin-like compounds in mammalian cells, with PGF_{2α} analogues being the most abundant form, while analogues of PGD₂ and PGE₂ are also found (Jahn et al., 2008) (Figure 2). Morrow and co-workers (1990) identified the production of a prostaglandin-like compound (8-epi-prostaglandin F_{2α}) through free radical catalyzed peroxidation of AA, independent of COX activity, in humans. These compounds are known as the isoprostanes, and even though they contain a cyclopentane prostane ring, similar to prostaglandins, they differ in side chain structures and stereochemistry (Milne et al., 2008). Isoprostane production has been discovered not only from AA, but also from adrenic acid [22:4(n-6)], DHA and EPA (Roberts & Milne, 2009).

Furthermore, HETEs can also be produced through free radical catalyzed lipid peroxidation, giving the same products as LOX or CYP450s (Buczynski et al., 2009) (Figure 2). Oxidative stress and these free radical-catalyzed reactions have been found in mammals as well as in plants, leading to the production of prostaglandin-like compounds called phytoprostanes from mono-, di- and tri-hydroxy-PUFAs (Mueller, 2004).

1.4.2. Fungal pathways

The previous information indicates that oxylipin production in mammalian cells has been well studied. This is in contrast to the limited information available on the mechanisms involved in oxylipin production in fungi, and the purpose of the next section will be to summarize our current knowledge regarding the production of oxylipins in fungi.

Enzymatic involvement in these pathways was indicated by incubating AA together with boiled lysates of either *C. albicans* or *Crypt. neoformans* (Erb-Downward et al., 2008; Erb-Downward & Huffnagle, 2007; Erb-Downward & Noverr,

2007). This led to a significant reduction in PGE₂ produced, suggesting the presence of a denaturable enzymatic pathway in these yeasts.

Brodhun and Feussner (2011) speculated about the unlikelihood of the existence of a specific prostaglandin pathway in fungi. They ascribed these reactions to be similar to a known isoprostane type of non-specific lipid peroxidation reaction that can be catalyzed by any protein harbouring iron as cofactor. This may not be as unlikely because this has already been found in humans, with the *in vivo* formation of PGF_{2α}-like compounds by free radical-catalyzed peroxidation of AA from phospholipids (Morrow et al., 1990, 1992). However, Erb-Downward and Noverr (2007) demonstrated that PGE₂ formed by *C. albicans* did not possess isoprostane stereochemistry, indicating that PGE₂ production was not due to non-specific lipid peroxidation in this yeast.

The use of different enzyme inhibitors is widely applied in order to identify the pathways and putative enzymes involved in eicosanoid production by fungi and a number of studies that used COX inhibitors, including ASA and other NSAIDs, as well as LOX inhibitors, were used to identify possible mechanisms involved in fungal eicosanoid production (Botha et al., 1997; Erb-Downward et al., 2008; Kock et al., 1992; Lamacka & Sajbidor, 1998), however this has not been able to provide conclusive evidence.

1.4.2.1. *Filamentous fungi*

Lipoxygenases are the first dioxygenases identified and characterized in several fungi, either directly by illustrating LOX activity, or indirectly via the addition of LOX inhibitors, or the presence of LOX-derived oxylipins. These fungi include the mesophilic fungus, *Fusarium oxysporum* (Bisakowski et al., 1995; Satoh et al., 1976), the dimorphic fungus, *Ceratocystis ulmi* (Jensen et al., 1992), the plant pathogen, *Geotrichum candidum* (Perraud et al., 1999), several *Penicillium* species (Perraud & Kermasha, 2000), the thermophilic soil fungus, *Thermomyces lanuginosus* (Li et al., 2001), as well as the baker's yeast, *S. cerevisiae* (Bisakowski et al., 1997; Shechter & Grossman, 1983). In *G. graminis*, a 18:2 (13*R*)-LOX which differed from mammalian and plant LOX, by having a manganese catalytic centre

and being secreted, was characterized, cloned and expressed (Su & Oliw, 1998). This LOX forms (13*R*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid from 18:2(n-6). In the oomycetous fungus, *S. parasitica*, a soluble LOX, which has a hydroperoxide isomerase activity, converting hydroperoxide into epoxy alcohols, was also purified (Hamberg, 1986; Herman & Hamberg, 1987). The search for LOX homologues in fungal genomic databases revealed the presence of fungal LOX with sequence similarity in *A. fumigatus*, *A. nidulans*, *A. oryzae*, *F. graminearum* and *Neurospora crassa* (Tsitsigiannis et al., 2005a). The different sources of LOX, contributes to its different substrate specificities as well as its activity at different pH ranges.

Additionally, a metalloenzyme, leukotriene A₄ hydrolase (LTA₄H), having both epoxide hydrolase and peptidase activity, was cloned, expressed and characterized in *S. cerevisiae* (Kull et al., 1999, 2001). This enzyme showed a 42% identity to human LTA₄H, known to hydrolyze LTA₄ to the pro-inflammatory mediator LTB₄. The *S. cerevisiae* LTA₄H has been shown to hydrolyze LTA₄, to LTB₄ as well as two other compounds [5*S*,6*S*-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (diHETE) and Δ^6 -*trans*- Δ^8 -*cis*-leukotriene B₄].

Research, including protein purification and gene cloning, has identified the presence of a number of CYP450 genes in filamentous fungi (Park et al., 2008). This includes approximately 4538 CYP450s in total in 66 fungal and four oomycete species. This is largely due to the increasing availability of fungal genome sequences. Cytochrome P450s are proposed to be responsible for the survival of fungi in different ecological habitats. A stable intracellular CYP450 with LOX activity, induced during growth on soybean oil as carbon source, was purified from *F. oxysporum* (Shoun et al., 1983). This CYP450 differed from other fungal CYP450s by being present in the soluble fraction and not membrane bound. Additionally, two CYP450 monooxygenases, responsible for the metabolism of 18:1(n-9) to ω -hydroxy fatty acids and finally to α,ω -dicarboxylic acids were expressed in *C. tropicalis* (Eschenfeldt et al., 2003). One of these CYP450s could also oxidize saturated fatty acids such as myristic acid (14:0).

In the fungus, *G. graminis*, the hydroxylation and epoxidation reactions leading to the production of HODE were proposed to be by CYP450, since the oxygenation reactions differed from normal LOX reactions (Brodowsky et al., 1992;

Brodowky & Oliw, 1992). One of these enzymes was purified to homogeneity and identified as the haemprotein, linoleate diol synthase (LDS), a dioxygenase which shares homology with COX, but which is not present in animals or plants (Hörnsten et al., 1999). A similar LDS was identified in the closely related fungus, *Magnaporthe grisea* (Cristea et al., 2003). The identification of LDS in certain fungi led to the identification of three COX-like dioxygenases which are structurally similar to mammalian COX in the opportunistic pathogens, *A. fumigatus* and *A. nidulans* (Tsitsigiannis et al., 2005a, b). These dioxygenases were identified as Ppo enzymes (psi-producing oxygenases) and are encoded by *ppoA*, *ppoB* and *ppoC* genes containing amino acid sequences with both oxygenase and peroxidase regions. These genes are responsible for the production of the oxylipins known as psi-factors, from 18:1(n-9) and 18:2(n-6). Interestingly, these *ppo* genes were also found to be involved in prostaglandin production from exogenous AA by *A. fumigatus* and *A. nidulans* (Tsitsigiannis et al., 2005b). Homologues of these genes were also present in both saprophytic and pathogenic Ascomycetes (i.e. *A. oryzae*, *F. graminearum*, *F. verticillioides*, *Histoplasma capsulatum*, *Magnaporthe grisea* and *N. crassa*) and Basidiomycetes (i.e. *Coprinus cinereus*, *Phanerochaete chrysosporium* and *Ustilago maydis*) (Tsitsigiannis et al., 2005a). However, no homologues of *ppo* genes are present in *Candida* or *Cryptococcus*, possibly being restricted to filamentous fungi.

1.4.2.2. *Dipodascopsis uninucleata*

The production of 3*R*-HETE in *D. uninucleata* was initially proposed to be by COX or LOX enzymes, through the use of different inhibitors. However, it is now believed that a partial β -oxidation process or a direct monooxygenase reaction by a CYP450 type enzyme is involved (Akpinar et al., 1998; Venter et al., 1997). This was indicated through the necessity for a 5*Z*,8*Z*-diene system needed for the hydroxylation of various fatty acids to produce 3-OH fatty acids by this yeast (Venter et al., 1997). This can be accomplished by incomplete β -oxidation or partial breakdown of the fatty acid, indicating the presence of a 2-enoyl-CoA hydratase or CYP450 type enzyme responsible for a monooxygenase or hydroxylase reaction.

Later, Fox and co-workers (2000a) indicated that this β -oxidation occurs in the mitochondria, and not in peroxisomes or specialized microbodies. This was indicated through the addition of mitochondrial β -oxidation cofactors such as ATP, NAD⁺ and Mg²⁺, which led to an increase in the production of 3R-HETE by cell-free enzyme extracts of this yeast, whereas in the absence of these cofactors no conversion of AA occurred. Another factor that contributed to this finding was the inhibition of 3R-HETE by antimycin A, an inhibitor of the oxidation of ubiquinol in the electron transport chain of oxidative phosphorylation in the mitochondria.

1.4.2.3. *Candida albicans*

Initially Noverr and co-workers (2001) speculated that COX-like enzymes had to be present in *C. albicans*. In their studies they used an ELISA assay and HPLC analysis with different COX inhibitors (i.e. etodolac, indomethacin and piroxicam), to evaluate prostaglandin production by these species. They found that all these inhibitors not only decreased prostaglandin production but also decreased the viability of these cells. This suggests that the decrease was not due to a specific inhibition of the enzyme but rather due to an effect on cell viability or that prostaglandin production could regulate the viability of the yeast (Erb-Downward & Huffnagle, 2007). Later, using non-selective mammalian COX inhibitors, ASA, indomethacin and resveratrol, and the LOX inhibitor, nordihydroguaiaretic acid (NDGA) (also known as a COX inhibitor) the production of PGE₂ was reduced without affecting viability (Erb-Downward & Noverr, 2007). However, the use of the selective COX-2 (PGHS-2) inhibitor, CAY10404 [3-(4-methylsulphonylphenyl)-4-phenyl-5-trifluoromethyl-isoxazole], had no effect on PGE₂ production, suggesting that enzymes distinct from mammalian COX and LOX are responsible for PGE₂ production in *C. albicans* (Erb-Downward & Noverr, 2007). This agrees with the BLAST results used to search the genome of *C. albicans* for COX and LOX homologues, which did not reveal any sequences with significant homology to mammalian COX and LOX (Haas-Stapleton et al., 2007; Tsitsigiannis et al., 2005a). This was followed by the identification of two non-COX/LOX-related enzymes, involved in PGE₂ production in *C. albicans* (Erb-Downward & Noverr, 2007). These enzymes were identified as a fatty acid desaturase homologue, Ole2p, and a

multicopper oxidase or laccase homologue, Fet3p. The importance of these genes was illustrated by indicating that mutants lacking the *ole2* or *fet3* gene had a reduced production of PGE₂. However, this did not completely inhibit PGE₂ production, suggesting that other enzymes are also involved. Additionally, CYP52A21, a putative fatty acid hydroxylase was characterized from *C. albicans* (Kim et al., 2007). This enzyme can hydroxylate fatty acids of different lengths at the ω -1 position.

The production of RvE1, as well as its precursors, 18-HEPE, 15-HEPE and 5-HEPE from EPA by *C. albicans*, differs from the mechanism in humans in that the production takes place independent from other cellular partners (Figure 3) (Haas-Stapleton et al., 2007). However, the enzymes involved are still unknown. Although it was speculated that LOX and CYP450 monooxygenases had to be present, due to the sensitivity of RvE1 production towards LOX and CYP450 inhibitors, this could not be confirmed.

1.4.2.4. *Cryptococcus neoformans*

Since the COX inhibitor, indomethacin, could reduce prostaglandin production in *Crypt. neoformans*, it was initially speculated that the enzyme involved was COX-related (Noverr et al., 2001). However, similar to *C. albicans*, the genome of *Crypt. neoformans* did not reveal any sequence homology to mammalian COX and LOX (Erb-Downward et al., 2008; Erb-Downward & Huffnagle, 2007) and a later study by Erb-Downward and Huffnagle (2007), did not observe this inhibitory effect in the presence of the COX inhibitors, ASA and indomethacin, suggesting that other non-COX enzymes are involved. It must, however, be noted that the difference in especially incubation time used in the latter study, may have contributed to the observed difference in results.

In another study, Erb-Downward and co-workers (2008) indicated that the polyphenolic LOX inhibitors, caffeic acid, NDGA and resveratrol, inhibited both PGE₂ and PGF_{2 α} production in *Crypt. neoformans*, even though a LOX homologue is absent. This led to the identification of laccase, a multicopper oxidase known to bind polyphenols, as an enzyme involved in prostaglandin production in *Crypt.*

neoformans (Figure 4). Laccase alone did not convert the PGE₂ precursors (AA or PGH₂) to PGG₂ or new prostaglandins, but it did convert PGG₂ to PGE₂ and 15-keto-PGE₂. This suggests that multicopper oxidases might play a significant role in eicosanoid production by this pathogenic yeast. However, it is not the only enzyme involved, and questions still need to be answered regarding the enzymes upstream of the multicopper oxidase. In addition, it has been speculated that enzymes belonging to the Old Yellow Enzyme (OYE) family might be involved in this pathway (Figure 4) (Erb-Downward et al., 2008).

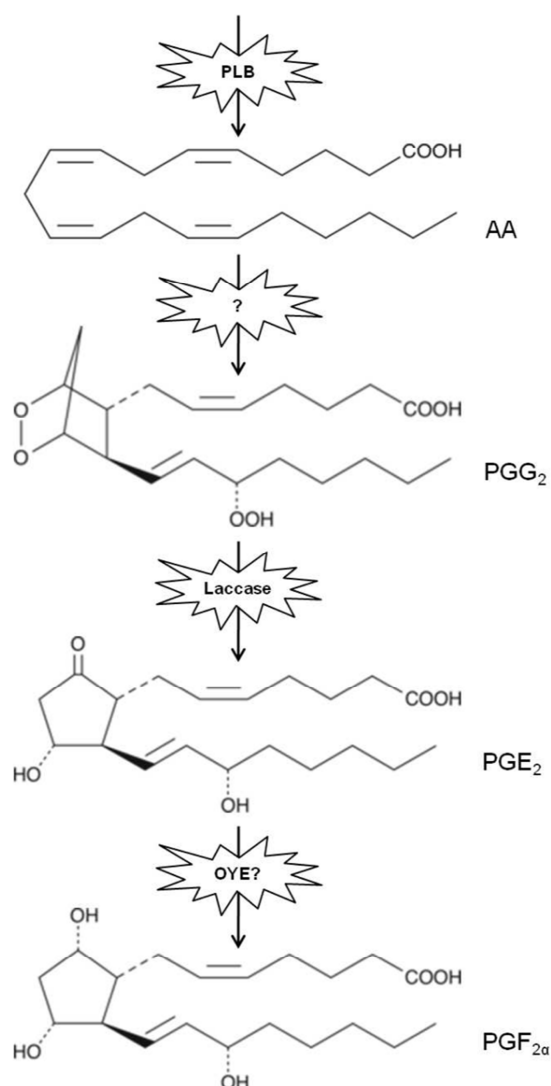


Figure 4. The role of laccase during prostaglandin production by *Cryptococcus neoformans* from arachidonic acid (AA). This also indicates the possible involvement of an Old Yellow Enzyme (OYE) in the production of PGF_{2α} from PGE₂. However, the enzymes involved in the production of PGG₂ from AA are still unknown. PLB: phospholipase B. (Erb-Downward et al., 2008).

1.4.2.5. *Paracoccidioides brasiliensis*

Similar to *C. albicans* and *Crypt. neoformans*, it is speculated that a COX pathway is involved in the production of PGE_x by the dimorphic fungus, *P. brasiliensis* (Biondo et al., 2010; Bordon et al., 2007). This was indicated by the use of indomethacin and piroxicam, which not only inhibited PGE_x production but also fungal viability. The authors suggest a COX-dependent metabolic pathway is involved and that, similar to *C. albicans*, prostaglandins have a possible role in fungal survival.

1.5. Biological activity of oxylipins

Eicosanoids are active at low nanomolar concentrations and have very short half-lives (Funk, 2001; Tsitsigiannis & Keller, 2007; Yilmaz, 2001). Therefore they act near the site of synthesis. Interestingly, prostaglandins are not stored in tissue lipids, as is the case with HETEs and EETs, but are synthesized immediately when needed (Carroll & McGiff, 2000). The production of eicosanoids by mammalian cells is in response to mechanical factors or chemical stimuli, such as cytokines, or in response to pathogen invasion (Funk, 2001). They act similar to hormones, as potent biological regulators and are involved in many systems such as the cardiovascular system, renal function as well as reproduction and the immune system (Goodwin & Ceuppens, 1983; Holland et al., 1988). The immunomodulatory properties of eicosanoids have been studied intensively in mammalian cells with a single eicosanoid capable of having pleiotropic functions (Funk, 2001; Hatae et al., 2002). This includes different physiological and pharmacological effects on different cell types. These effects are mainly due to the existence of multiple receptors for each lipid species on plasma membranes. Eicosanoids are known to function through G-protein-coupled receptors (GPCRs), known as guanine nucleotide regulatory proteins, to elicit their pharmacological and signalling profiles (Smith, 1989). The activated trimeric G-proteins affect the concentrations of the second messengers, cyclic AMP (cAMP), or intracellular ions such as K⁺. This occurs through the stimulation or inhibition of adenylate cyclase or the opening or closing of K⁺ channels.

1.5.1. Prostanoids

Prostanoids have a wide variety of tissue-dependent functions in biological systems including vasodilation, platelet aggregation and pain induction (Funk, 2001; Hata & Breyer, 2004; Newton & Roberts, 1997; Yilmaz, 2001). The prostanoids are known to be recognized by nine receptors, a subfamily of GPCRs i.e. chemoattractant receptor-homologous molecule expressed on T helper cell type 2 (Th2) (CRTH2), D prostanoid receptor (DP), E prostanoid receptor (EP1-4), F prostanoid receptor (FP), prostacyclin receptor (IP) and thromboxane receptor (TP) (Hata & Breyer, 2004). Prostaglandin E₂ is known to act via four different receptors known as EP1-4 (McCoy et al., 2002) providing it with both anti- and pro-inflammatory responses (Hata & Breyer, 2004). Prostaglandin E₂ elicits its anti-inflammatory effects by inhibiting mediator release from phagocytes, T helper cell type 1 (Th1) immune responses, chemokine production, macrophage phagocytosis and dendritic cell functions (Aronoff et al., 2004; Betz & Fox, 1991; Harizi & Gualde, 2006; van der Pouw Kraan et al., 1995). These effects are believed to be due to the increase in cAMP levels. The inhibition of Th1 responses by PGE₂ is due to the inhibition of IL-12, which selectively up-regulates IFN- γ production, the cytokine responsible for inducing Th1 responses (van der Pouw Kraan et al., 1995). Fabricius and co-workers (2010) indicated the inhibition of dendritic cell derived IFN- α by PGE₂ followed by a suppression of Th1 cytokine production. Additionally, PGE₂ was also found to enhance tissue eosinophilia and Th2 responses through IL-6 and IL-10 production during inflammation (Fabricius et al., 2010; Harizi et al., 2002; Kalinski et al., 1997; McCoy et al., 2002; Profita et al., 2003). Interleukin-6 is also one of the cytokines responsible for inducing the newly identified Th17 cells, to produce IL-17 during infection (Bettelli et al., 2007; van der Meer et al., 2010; Weaver et al., 2007). These Th17 cells play an important part in providing protection against bacteria and fungi. However, pro-inflammatory activities also depend on the prostaglandin receptors mediating different signal transduction pathways (Funk, 2001; Hata & Breyer, 2004). Pro-inflammatory activities involve the activation of the innate immune response, a non-specific reaction and the first layer of defence against pathogens. Prostacyclin (PGI₂) acting via the IP receptor, enhances vasodilation in coronary blood vessels and is involved in platelet declumping (Funk, 2001). This is in contrast to the thromboxane, TXA₂, acting via the TP receptor (Hata & Breyer, 2004) which is

associated with the constriction of blood vessels and promotes platelet aggregation (Funk, 2001). This indicates the opposing effects of prostanoids during inflammation. Other important prostaglandins are $\text{PGF}_{2\alpha}$, acting via the FP receptor which is involved in the contraction of uterine smooth muscle, PGD_2 acting via the DP and CRTH2 receptors, which enhances Th2 lymphocyte chemotaxis and causes the allergic reactions in lung epithelial cells leading to asthma (Funk, 2001; Hata & Breyer, 2004).

1.5.2. LOX products

Leukotrienes are associated with pro-inflammatory functions and implicated in the pathogenesis of several inflammatory diseases, such as asthma, inflammatory bowel disease, psoriasis and rheumatoid arthritis (Henderson, 1994). Leukotriene B_4 plays a major part in neutrophil chemotaxis, aggregation and adhesion to endothelial and inflammatory cells, by up-regulating adhesins, during inflammation (Funk, 2001; Henderson, 1994). This leukotriene also increases the production of certain cytokines, such as IL-6 and IL-2, enhances T-lymphocyte proliferation, and plays a role in inflammatory pain, upregulating phagocytosis and the recruitment of leukocytes (Henderson, 1994). The other leukotrienes, referred to as sulfidopeptide leukotrienes or cysteinyl leukotrienes, LTC_4 , LTD_4 and LTE_4 , act as immunomodulatory agents, increase vasoconstriction, vasopermeability, mucous secretion, attract eosinophils and are involved in smooth-muscle contractions such as bronchoconstriction (Funk, 2001; Henderson, 1994). In addition, the HETEs and the hepoxilins are involved, amongst others, in the activation of mitogen protein kinase signalling, the expression of monocyte chemoattractant protein-1, angiogenesis, cancer growth, neuronal apoptosis, intracellular calcium release and neutrophil migration (Buczynski et al., 2009).

The other LOX related products, the lipoxins, were the first compounds identified with pro-resolving actions *in vivo* (Serhan et al., 2008b). They demonstrate anti-inflammatory activity, promoting the resolution of inflammation, which mediates the return to tissue homeostasis, preventing chronic inflammation, which could lead to inappropriate tissue damage. These compounds stop the entry of neutrophils to

the sites of inflammation, reduce vascular permeability, regulate chemokine production and reactive oxygen species (ROS) and down-regulate T-lymphocyte responses (Rajakariar et al., 2006; Serhan et al., 2008b).

1.5.3. Cytochrome P450-derived eicosanoids

The eicosanoid products of CYP450 have vasoactive activities, acting as vasoconstrictors and vasodilators. The CYP450 ω -hydroxylase produce HETEs, primarily known as vasoconstrictors and are involved in the regulation of smooth muscle responses and are considered important in pulmonary function (Carroll & McGiff, 2000). 20-Hydroxy eicosatetraenoic acid promotes systemic vasoconstriction in canine renal arcuate arteries (Ma et al., 1993), constricts pulmonary arteries in newborn piglets (Fuloria et al., 2004) and promotes vasoconstriction in hypertensive rat mesenteric vessels (Chu et al., 2000). Additionally, 20-HETE is the most abundant eicosanoid produced by the kidney and believed to be involved in the disturbances of renal functions in patients with hepatic cirrhosis (Carroll & McGiff, 2000). In contrast, 18-HETE and 19-HETE have an opposing effect, promoting vasodilation (Carroll et al., 1996). In addition, 16-HETE is important during inflammation and inhibits neutrophil adhesion and the activation of PMN leukocytes (Bednar et al., 2000).

The EETs (11,12- and 14,15-EET), products of CYP450 epoxidases, are primarily known as vasodilators and play an important role during inflammation, control renal function and neuronal signalling (Buczynski et al., 2009; Larsen et al., 2008). They also decrease platelet aggregation, cytokine-induced endothelial cell adhesion and prevent leukocyte adhesion to the vascular wall. In contrast, they can also have opposing effects during vascular inflammation e.g. having a non-vasodilatory role (e.g. 5,6-EET) (Node et al., 1999).

1.5.4. Non-enzymatically-derived eicosanoids

The isoprostane from free radical catalyzed peroxidation of AA, 8-epi-prostaglandin F_{2 α} , also showed biological activity by reducing renal blood flow,

glomerular filtration rate and vasoconstriction (Morrow et al., 1990). This isoprostane can also be used to measure endogenous oxidant stress (Roberts & Milne, 2009). The increased occurrence of isoprostanes during oxidative stress could emphasize its biological importance in oxidant injury.

1.5.5. Fungal-derived oxylipins

It is known that CD4⁺ T cells differentiate into CD4⁺ Th cells, Th1 or Th2, in response to antigens or cytokines to eliminate pathogens (Sornasse et al., 1996). These Th cells secrete different cytokines and have different functions during an immune response. The Th1-type cytokines are involved in activating macrophages and cytotoxic T cells, known as cell-mediated/protective immunity against intracellular pathogens (Delves & Roitt, 2000). In addition, the Th2-type cytokines are involved in humoral immunity by helping B cells to produce antibodies. However, an imbalance in Th1/Th2 responses can be detrimental to the host, leading to serious autoimmune diseases while these responses can also negatively regulate each other (van der Pouw Kraan et al., 1995). Interestingly, pathogen studies suggested that eicosanoid production, especially PGE₂, could shift these Th responses in favour of the pathogen (Shibata et al., 2005; van der Pouw Kraan et al., 1995). Therefore, the activation of Th1 and/or Th2 responses may correlate to the occurrence of resistance and susceptibility to infections.

Both host and pathogen are capable of producing PGE₂ during an infection indicating that both can modulate immune responses (Betz & Fox, 1991; Rocco & FitzGerald, 2002; Shibata et al., 2005). These biological effects of prostaglandins on the immune system and the enhanced production of prostaglandins and leukotrienes by pathogenic fungi, may lead to the intracellular survival followed by chronic and disseminated infections (Noverr et al., 2003). This can be illustrated by the ability of these eicosanoids to down-regulate macrophage functions as mentioned above for mammalian eicosanoids.

Candida infections stimulate both the innate and adaptive immune responses (Romani, 2000; Santoni et al., 2002; Schaller et al., 2002). Although several studies indicated that *C. albicans* and *Crypt. neoformans* stimulate the production of Th1-

type cytokines leading to protective immunity or acquired resistance against these pathogens (Cenci et al., 1995; Montagnoli et al., 2004; Romagnoli et al., 2004; Schaller et al., 2002; Traynor et al., 2000), the production of PGE₂ during infections inhibits Th1 responses and has an inducing or no effect on Th2 responses (Betz & Fox, 1991; Osterholzer et al., 2009). Similarly, Noverr and co-workers (2001) indicated that, *C. albicans* PGE₂, has immunosuppressive effects in mammalian cells by down-modulating chemokine production, tumor necrosis factor alpha (TNF α) production and splenocyte proliferation while up-regulating IL-10 production (Th2 responses). It is known that Th1 responses are critical for protection against candidiasis whereas Th2 responses are less critical and non-protective against pathogens leading to chronic or disseminated infections (Navarathna et al., 2007a; Romani, 2000). So the production of PGE₂ during infections might be beneficial for the pathogens. Another important factor induced by PGE₂, is tissue eosinophilia, leading to tissue damage, which is a common feature of some chronic fungal infections (Betz & Fox, 1991). In addition, it is known that *C. albicans* PGE₂ enhances TH17 responses by stimulating the production of IL-17 and IL-22 (Smeekens et al., 2010).

As mentioned above, *C. albicans* is also capable of producing RvE1 from EPA (Haas-Stapleton et al., 2007). The production of RvE1 at low concentrations dampens the host's immune response which will allow the continuation of commensal *C. albicans* inside the host. In contrast, it was found that, at high RvE1 concentrations, the resolution of inflammation was not as effective compared to low RvE1 concentrations. This indicates that the host immune response in heavily colonized tissue will not be dampened.

The biological activity of the purified PGE_x (PGE₂) on yeast was indicated through the stimulation of germination in *C. albicans*, similar to synthetic PGE₂ and thromboxane B₂ (TXB₂) (Noverr & Huffnagle, 2004). This was illustrated through the inhibition of germ tube formation when antibodies against PGE₂ and TXB₂ were added. Similar results were obtained by Kalo-Klein and Witkin (1990) using commercial PGE₂, suggesting that morphogenesis (yeast-to-hyphae transition) is induced in *C. albicans* by a PGE₂ caused increase in cAMP levels. The increase in intracellular cAMP levels in guinea-pig tracheal epithelial cells due to commercial

PGE₂ was also observed (Pelletier et al., 2001). However, other prostaglandins i.e. PGD₂, PGF₂ and PGI₂, did not exert any effect on cAMP. Interestingly, PGF_{2α} had no effect on germination of *C. albicans* (Kalo-Klein & Witkin, 1990). This suggests that the other prostaglandins, PGD₂ and PGI₂, may also not affect germination by *C. albicans*. The PGE₂ precursor, AA as well as other long chain fatty acids (i.e. 18-carbon fatty acids) did not have any effect on germination, while the short chain fatty acid, butyric acid (4:0), inhibited germination (Noverr & Huffnagle, 2004).

Morphogenesis in pathogenic fungi is associated with increased virulence and mucosal invasiveness (Rooney & Klein, 2002) and the start of biofilm formation, with infections by *C. albicans* mainly caused by biofilms (Ramage et al., 2005). This suggests that prostaglandin production, especially PGE₂, might be an important virulence factor. However, the exact role of prostaglandins in morphogenesis and biofilm development is complex and remains unclear. Although, Alem & Douglas (2005) indicated the production of PGE₂ by *C. albicans* biofilms, PGE₂ did not function as a QS molecule, with no correlation found between PGE₂ production and cell density.

However, the oxylipin farnesol, specifically E,E-farnesol, functions as a QS signal of *C. albicans* and *C. dubliniensis* (Henriques et al., 2007; Hornby et al., 2001). This compound inhibits morphogenesis and biofilm formation during high-density growth, allowing the culture to grow as actively budding yeasts (Kruppa et al., 2004; Ramage et al., 2002). The QS mechanism of farnesol in *C. albicans* relies on inhibition of mitogen-activated protein kinase (MAPK) cascades through the suppression of *HST7* and *CPH1* (Sato et al., 2004). In another study, it was indicated that the cAMP-protein kinase A pathways, responsible for hyphal formation, is inhibited by farnesol through the inhibition of the Ras1-Cdc35 pathway (Davis-Hanna et al., 2008). Farnesol also up-regulated the hyphal-suppressor genes, *TUP1* and *HOG1*, and down-regulated the morphogenesis associated genes, *CRK1* and *PDE2* (Cao et al., 2005; Kebaara et al., 2008; Smith et al., 2004).

Farnesol also plays a role during *C. albicans* infections in host pathogen interactions. Ghosh and co-workers (2010) indicated in murine RAW264.7 macrophages that farnesol, produced by *C. albicans* during infection, together with zymosan, a yeast cell wall preparation that contains pathogen-associated molecular

patterns, stimulated the production of inflammatory and regulatory cytokines (e.g. IL-1 β and TNF- α). Décanis and co-workers (2009) found that exogenously added farnesol increased protection of epithelial cells (engineered human oral mucosa tissue) against *C. albicans* infection through the increased production of TLR2 and human β -defensin 2. In contrast, it was found that farnesol is also an important virulence factor in *C. albicans*. This was illustrated by a *C. albicans* mutant strain, from which phosphatase was deleted (Navarathna et al., 2007b). This enzyme is known to be responsible for metabolizing farnesyl pyrophosphate to farnesol. As expected the mutant strain produced less farnesol and was less pathogenic in a mouse infection model. Similarly, when these mice were exogenously fed or injected with farnesol followed by infection with *C. albicans*, pathogenesis and mortality was increased. The results indicated an enhanced production of the Th2-type cytokine, IL-5, and a reduction in Th1-type cytokines, IFN- γ and IL-12 (Navarathna et al., 2007a).

Farnesol was also found to protect *C. albicans* cells against oxidative stress (Westwater et al., 2005). The production of farnesol by *C. albicans* might also help *C. albicans* to compete with other fungi in a mixed population. *Aspergillus nidulans*, which does not produce farnesol, responds to external farnesol by undergoing apoptosis, without affecting *A. nidulans* hyphal morphogenesis (Semighini et al., 2006).

Additionally, Nigam and co-workers (2011) indicated the ability of 3(*R*)-HTDE, a β -oxidation product of the *C. albicans* endogenous fatty acid, 18:2(n-6), to act as QS molecule in *C. albicans*. They found that 18:2(n-6) and 3(*R*)-HTDE enhanced germ tube formation with only the 3(*R*)-HTDE capable of enhancing biofilm formation in *C. albicans*. Interestingly, the biofilm inhibitory effect of farnesol was reduced in the presence of 3(*R*)-HTDE. However, although the exact mechanism involved is still under investigation, it is believed that two unknown dihydroxyl molecules from 3(*R*)-HTDE metabolism, detected by HPLC analysis, might also be involved.

As mentioned before, *C. albicans* is capable of synthesizing 3,18-diHETE from exogenous AA (Deva et al., 2000). It was shown, through immunofluorescence microscopy and a polyclonal antibody, specific against 3-OH fatty acids (Bhatt et al., 1998), that 3,18-diHETE is mainly associated with the hyphal forms of *C. albicans*

and not the yeast form (Figure 5) (Deva et al., 2000, 2001). This might play a role in the anchorage of these cells to host cells during infections. This indicates that 3-OH fatty acid production may be an important virulence factor in *C. albicans* as well as in *C. dubliniensis*, since 3,18-diHETE production has also been found in this closely related yeast (Ells, 2008).

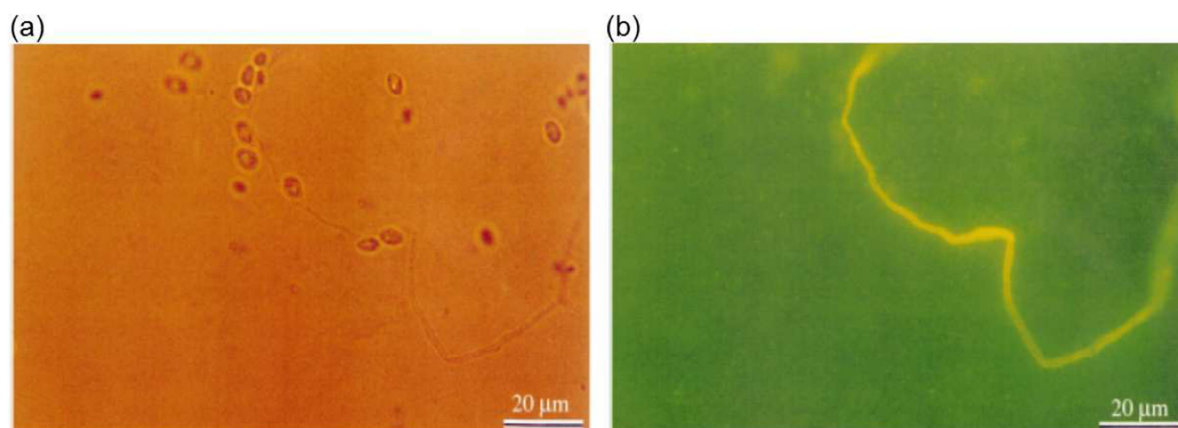


Figure 5. (a) Light micrograph of *Candida albicans* indicating the hyphae surrounded by yeast-like budding cells. (b) The same area is shown with an epifluorescence device and only the hyphae and not the budding cells can be seen (the antibody against 3-HETE was used for the detection) (Deva et al., 2000).

3*R*-HETE, produced by the non-pathogenic yeast *D. uninucleata*, was also reported to have pro-inflammatory actions in mammalian cells by affecting signal transduction processes in human neutrophils and tumour cells (Nigam et al., 1996, 1999). Ciccoli and co-workers (2005) indicated that 3-HETE, similar to that produced by *D. uninucleata*, can be used as a substrate for *C. albicans* activated mammalian COX-2, in a HeLa infection model (Deva et al., 2003), to produce pro-inflammatory 3-OH PGE₂. This metabolite increased inflammation in host cells. The immunomodulatory activities of this compound were illustrated through the increased up-regulation of IL-6 gene expression (an enhanced Th2 response), in comparison to PGE₂, as well as cAMP levels, similar to PGE₂, in Jurkat T-cells and lung adenocarcinoma cells (A549 cells), respectively (Betz & Fox, 1991; Ciccoli et al., 2005).

In *D. uninucleata* and *D. tothii*, 3*R*-HETE is mainly associated with morphogenesis, sexual spore formation and the release of ascospores from the ascus by acting as possible lubricants, as well as the arrangement of ascospores after release (Coetzee et al., 1992; Kock et al., 1998; Smith et al., 2000; van Dyk et al., 1991). This suggests a possible role in the life cycle of yeasts (Figure 6). Interestingly, in *D. tothii*, oxylipins were only found on the tips of the empty asci, in contrast to *D. uninucleata* where the oxylipins are mainly present between and on the surface of the released ascospores.

This led to the discovery of 3-OH fatty acid association with the surface structures of ascospores and asci of many types of yeast (Kock et al., 2003). However, it is important to note that most of these 3-OH fatty acids are not produced from exogenous AA but rather from other precursors. Interestingly, by using polyclonal antibodies together with immunofluorescence microscopy and GC-MS, the presence of 3-OH 8:0 on protruding cell surface structures of *S. cerevisiae*, was indicated (Kock et al., 2000). Strauss and co-workers (2005) indicated that ASA inhibition of 3-OH 8:0 production led to a reduction in flocculation in this yeast. This indicates the possible involvement of these 3-OH fatty acids in flocculation, an important step during the brewing process to separate yeasts to produce a good quality product (Verstrepen et al., 2003).

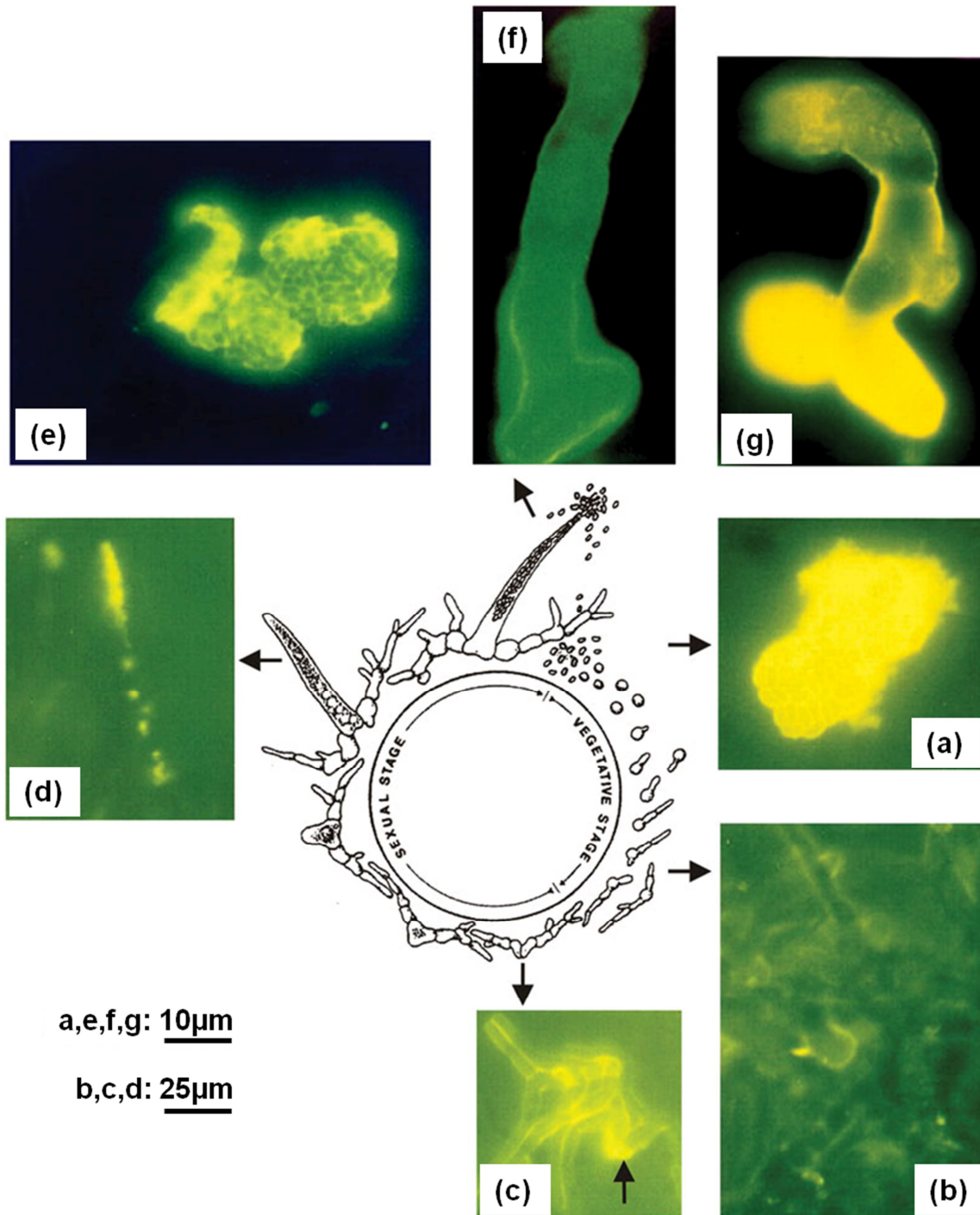


Figure 6. The distribution of 3R-HETE in the life cycle of *Dipodascopsis uninucleata* as visualized by immunofluorescence mapping. (a) Released ascospores. (b) Hyphae with cell wall. (c) Gametangiogamy. (d) Young ascus with cell wall. (e) Released ascospores from ascus with cell wall. (f) Empty ascus protoplast: still with characteristic morphology i.e. base (bottom) and shaft. (g) Deformed mature ascus protoplast containing ascospores mainly at base (Kock et al., 1998).

In *Saccharomycopsis malanga*, the production of 3-OH 16:0 was indicated (Kurtzman et al., 1974), and by using electron microscopy it was found that this 3-OH fatty acid is associated with the outer surface of the vegetative yeast cells forming thread-like structures (Sebolai et al., 2001). This can possibly be involved in the aggregation of the yeast cells. In the plant pathogen, *Eremothecium sinECAUDUM*, 3-OH fatty acids were found to be associated with ascospores (Bareetseng et al., 2004). This discovery made it possible for the researchers to identify a possible method of ascospore release from oval-shaped asci, suggesting that 3-OH fatty acids act as lubricants during ascospore release. Similar results were obtained for other *Eremothecium* spp. (Leeuw et al., 2007) as well as for *Dipodascus* spp. (Van Heerden et al., 2005, 2007) and *Ascoidea* spp. (Ncango et al., 2006). In the human pathogen, *Crypt. neoformans*, a 3-OH fatty acid, identified as 3-OH 9:1, was found to accumulate in capsules and to be released through protuberances as hydrophobic droplets (Sebolai et al., 2007). The exact function of these 3-OH fatty acids is still unknown, but it is speculated to play an immunomodulatory role during *Crypt. neoformans* infections, or to have a protective effect during fungal competition, but this needs further evaluation.

Additionally, in *A. nidulans* the addition of AA induced the formation of conidia, i.e. asexual sporulation, whereas the addition of PGE₂ inhibited conidiation (Tsitsigiannis et al., 2005b). However, in *A. fumigatus*, neither AA nor PGE₂ had an effect on conidiation. As indicated before, *A. nidulans* is capable of producing the oxylipins known as psi factors. These oxylipins were found to regulate both asexual and sexual spore development in this fungus (Champe & El-Zayat, 1989). In *A. nidulans*, the oxylipins derived from 18:2(n-6), psiB α and psiC α , stimulates sexual spore (ascospore) formation and inhibits asexual spore (conidia) development (Champe et al., 1987; Champe & El-Zayat, 1989). In contrast, psiA α had the opposite effect as observed for the above oxylipins. This indicates that the ratio of these oxylipins towards each other regulates asexual to sexual development in *A. nidulans* (Tsitsigiannis et al., 2004).

1.6. Conclusions

This review indicates that fungal oxylipins are widely distributed in nature, even though only a small percentage of the fungal kingdom has been studied. The limited available knowledge about the possible pathways or enzymes involved in fungal oxylipin production compared to mammalian cells, is evident. This together with the fact that what is known about these pathways is often speculative, indicates the complexity of this biosynthesis by these lower organisms.

Regarding the use of COX and LOX inhibitors to identify pathways involved, it should be noted that these inhibitors are non-selective, therefore they could inhibit various processes needed for fungal viability, thus leading to cell death. The so called non-prostaglandin mediated effects by different NSAIDs have been indicated before in mammalian cells (Brooks & Day, 1991). These include the interactions of NSAIDs with biological membranes and their influence on important cell functions such as transmembrane anion transport, oxidative phosphorylation, enzyme activity (NADPH oxidase, phospholipase C), the uptake of AA as well as the inhibition of glucose-6-phosphate dehydrogenases (Asensio et al., 2007; Brooks & Day, 1991). These mechanisms, together with the effect of ASA on the mitochondrial activity of various yeasts, which emphasized the use of ASA as a possible antifungal (Kock et al., 2007), might provide a possible explanation for the observed effects in the different studies. Additionally, some of these inhibitors used, such as NDGA, also have antioxidative properties which could also lead to the observed decrease in prostaglandin production (Strong et al., 2008).

The statement that PGE₂ is responsible or involved in fungal viability should be interpreted with care. However, Alem and Douglas (2004) indicated that biofilm formation by *C. albicans* can be inhibited by low concentrations of ASA, but when ASA was added together with PGE₂, this inhibition was abolished. The exact role of PGE₂ during *C. albicans* biofilm formation is however still unclear and needs further investigation. Recently, de Quadros and co-workers (2011) found similar results when they indicated *C. albicans* growth inhibition by the COX inhibitors ASA, ibuprofen, indomethacin and sodium salicylate, but that the addition of PGE₂ partly or completely abolished the inhibiting effect of ASA, indomethacin and sodium salicylate.

The importance of eicosanoids during host pathogen interactions was also emphasized. More specifically, the effect on the immune response, the system that is responsible for protecting us against pathogens, can be altered through the production of eicosanoids. The production of these lipid mediators functions as components of a complex chemical signalling system between host and pathogen. Additionally, the effects of prostaglandins and its fatty acid precursors might be species specific, but that it does play an important role during pathogenesis cannot be ignored.

1.7. References

- Akpinar A, Fox SR, Ratledge C & Friend J. (1998). Biotransformation of arachidonic acid and other eicosanoids by the yeast *Dipodascopsis uninucleata*, the Oomycete fungi *Saprolegnia diclina* and *Leptomitus lacteus* and the Zygomycete fungus *Mortierella isabellina*. Recent Advances in Prostaglandin, Thromboxane, and Leukotriene research. Plenum Press, New York and London. pp 231-234.
- Alem MAS & Douglas LJ. (2004). Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. Antimicrob Agents Chemother 48: 41-47.
- Alem MAS & Douglas LJ. (2005). Prostaglandin production during growth of *Candida albicans* biofilms. J Med Microbiol 54: 1001-1005.
- Arita M, Bianchini F, Aliberti J, Sher A, Chiang N, Hong S, Yang R, Petasis NA & Serhan CN. (2005). Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. J Exp Med 201: 713-722.
- Aronoff DM, Canetti C & Peters-Golden M. (2004). Prostaglandin E₂ inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. J Immunol 173: 559-565.

- Asensio C, Levoine N, Guillaume C, Guerquin M-J, Rouguie K, Chrétien F, Chapleur Y, Netter P, Minn A & Lapique F. (2007). Irreversible inhibition of glucose-6-phosphate dehydrogenase by the coenzyme A conjugate of ketoprofen: A key to oxidative stress induced by non-steroidal anti-inflammatory drugs? *Biochem Pharmacol* 73: 405-416.
- Bagga D, Wang L, Farias-Eisner R, Glaspy JA & Reddy ST. (2003). Differential effects of prostaglandin derived from n-6 and n-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *P Natl Acad Sci* 100: 1751-1756.
- Bareetseng AS, Kock JLF, Pohl CH, Pretorius EE, Botes PJ, Van Wyk PWJ & Nigam S. (2005). The presence of novel 3-hydroxy oxylipins on surfaces of hat-shaped ascospores of *Ascoidea Africana* Batra & Francke – Grosmann. *Can J Microbiol* 51: 99-103.
- Bareetseng AS, Kock JLF, Pohl CH, Pretorius EE, Strauss CJ, Botes PJ, Van Wyk PWJ & Nigam S. (2004). Mapping 3-hydroxy oxylipins on ascospores of *Eremothecium sinicaudum*. *Antonie van Leeuwenhoek* 86: 363-368.
- Bareetseng AS, Kock JLF, Pohl CH, Pretorius EE, Strauss CJ, Botes PJ, Van Wyk PWJ & Nigam S. (2006). Mapping the distribution of 3-hydroxy oxylipins in the ascomycetous yeast *Saturnispora saitoi*. *Syst Appl Microbiol* 29: 446-449.
- Bednar MM, Gross CE, Russell SR, Fuller SP, Ahern TP, Howard DB, Falck JR, Reddy KM & Balazy M. (2000). 16(*R*)-hydroxyeicosatetraenoic acid, a novel cytochrome P450 product of arachidonic acid, suppresses activation of human polymorphonuclear leukocyte and reduces intracranial pressure in a rabbit model of thromboembolic stroke. *Neurosurgery* 47: 1410-1418.
- Berger A & Jomard A. (2001). Fatty acid uninterrupted by a methylene as anti-inflammatory agents in superficial tissues of mammals. Patent no. US 6,280,755 B1.
- Berger A, Monnard I, Baur M, Charbonnet C, Safonova I Jomard A. (2002). Epidermal anti-inflammatory properties of 5,11,14 20:3: Effects on mouse ear

- edema, PGE₂ levels in cultured keratinocytes, and PPAR activation. *Lipids Health Dis* 1: 1-12.
- Bettelli E, Korn T & Kuchroo VK. (2007). Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 19: 652-657.
- Betz M & Fox BS. (1991). Prostaglandin E₂ inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol* 146: 108-113.
- Bhatt RK, Falck JR & Nigam S. (1998). Enantiospecific total synthesis of a novel arachidonic acid metabolite 3-hydroxy eicosatetraenoic acid. *Tet Lett* 39: 249-252.
- Biondo GA, Dias-Melicio LA, Bordon-Graciani AP, Acorci-Valério MJ & Soares AMVC. (2010). *Paracoccidioides brasiliensis* uses endogenous and exogenous arachidonic acid for PGE_x production. *Mycopathologia* 170: 123-130.
- Bisakowski B, Kermasha S & Klopfenstein ML. (1995). Partial purified lipoxygenase from *Fusarium oxysporum*: Characterization and kinetic studies. *Process Biochem* 30: 261-268.
- Bisakowski B, Perraud X & Kermasha S. (1997). Characterization of hydroperoxides and carbonyl compounds obtained by lipoxygenase extracts of selected microorganisms. *Biosci Biotech Biochem* 61: 1262-1269.
- Bordon AP, Dias-Melicio LA, Acorci MJ, Biondo GA, Fecchio D, Peraçoli MTS & Soares AMVC. (2007). Prostaglandin E₂ production by high and low virulent strains of *Paracoccidioides brasiliensis*. *Mycopathologia* 163: 129-135.
- Botha A & Kock JLF. (1993). The distribution and taxonomic value of fatty acids and eicosanoids in the Lipomycetaceae and Dipodascaceae. *Antonie van Leeuwenhoek* 63: 111-123.
- Botha A, Kock JLF & Nigam S. (1997). The production of eicosanoid precursors by mucoralean fungi. *Adv Exp Med Biol* 433: 227-229.

- Botha A, Kock JLF, van Dyk MS, Coetzee DJ, Augustyn OPH & Botes PJ. (1993). Yeast eicosanoids. IV. Evidence for prostaglandin production during ascosporeogenesis by *Dipodascopsis tothii*. *Syst Appl Microbiol* 16: 159-163.
- Brash AR. (2001). Arachidonic acid as a bioactive molecule. *J Clin Invest* 107: 1339-1345.
- Brodhun F & Feussner I. (2011). Oxylipins in fungi. *FEBS J* 278: 1047-1063.
- Brodowsky ID, Hamberg M & Oliw EH. (1992). A linoleic acid (8*R*)-dioxygenase and hydroperoxide isomerase of the fungus *Gaeumannomyces graminis*. *J Biol Chem* 267: 14738-14745.
- Brodowsky ID & Oliw EH. (1992). Metabolism of 18:2, 18:3, 20:4 and 20:5 by the fungus *Gaeumannomyces graminis*. *Biochem Biophys Acta* 1124: 59-65.
- Brooks PM & Day RO. (1991). Nonsteroidal antiinflammatory drugs-differences and similarities. *Engl J Med* 324: 1716-1725.
- Buczynski MW, Dumlao DS & Dennis ED. (2009). Thematic Review Series: Proteomics. An integrated omics analysis of eicosanoid biology. *J Lipid Res* 50: 1015-1038.
- Cao YY, Cao YB, Xu Z, Ying K, Li Y, Xie Y, Zhu ZY, Chen WS & Jiang YY. (2005). cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. *Antimicrob Agents Chemother* 49: 584-589.
- Capdevila JH, Falck JR & Harris RC. (2000). Cytochrome P450 and arachidonic acid bioactivation: molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* 41: 163-181.
- Carroll J, Fearnley IM, Shannon RJ, Hirst J & Walker JE. (2003). Analysis of the subunit composition of complex I from bovine heart mitochondria. *Mol Cell Proteomics* 2: 117-126.

- Carroll MA, Balazy M, Margiotta P, Huang DD, Falck JR & McGiff JC. (1996). Cytochrome P-450-dependent HETEs: profile of biological activity and stimulation by vasoactive peptides. *Am J Physiol* 271: R863-R869.
- Carroll MA & McGiff JC. (2000). A new class of lipid mediators: cytochrome P450 arachidonate metabolites. *Thorax* 55: S13-S16.
- Cenci E, Mencacci A, Spaccapelo R, Tonnetti L, Mosci P, Enssle KH, Puccetti P, Romani L & Bistoni F. (1995). T helper cell type 1 (Th1)- and Th2-like responses are present in mice with gastric candidiasis but protective immunity is associated with Th1 development. *J Infect Dis* 171: 1279-1288.
- Champe SP & El-Zayat AAE. (1989). Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. *J Bacteriol* 171: 3982-3988.
- Champe SP, Rao P & Chang A. (1987). An endogenous inducer of sexual development in *Aspergillus nidulans*. *J Gen Microbiol* 133: 1383-1387.
- Chu ZM, Croft KD, Kingsbury DA, Falck JR, Reddy KM & Beilin LJ. (2000). Cytochrome P450 metabolites of arachidonic acid may be important mediators in angiotensin II-induced vasoconstriction in the rat mesentery *in vivo*. *Clin Sci* 98: 277-282.
- Ciccoli R, Sahi S, Singh S, Prakash H, Zafiriou MP, Ishdorj G, Kock JLF & Nigam S. (2005). Oxygenation by COX-2 (cyclo-oxygenase-2) of 3-HETE (3-hydroxyeicosatetraenoic acid), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3- hydroxyeicosanoids. *J Biochem* 390: 737-747.
- Coetzee DJ, Kock JLF, Botha A, van Dyk MS, Smit EJ, Botes PJ & Augustyn OPH. (1992). The distribution of arachidonic acid metabolites in the life cycle of *Dipodascopsis uninucleata*. *System Appl Microbiol* 15: 311-318.
- Cristea M, Osbourn AE & Oliw EH. (2003). Linoleate diol synthase of the rice blast fungus *Magnaporthe grisea*. *Lipids* 38: 1275-1280.
- Culp BR, Titus BG & Lands WEM. (1979). Inhibition of prostaglandin biosynthesis by eicosapentaenoic acid. *Prostaglandins Med* 3: 269-278.

- Davis-Hanna A, Piispanen AE, Stateva LI & Hogan DA. (2008). Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. *Mol Microbiol* 67: 47-62.
- Dècanis N, Savignac K & Rouabhia M. (2009). Farnesol promotes epithelial cell defense against *Candida albicans* through Toll-like receptor 2 expression, interleukin-6 and human Î2-defensin 2 production. *Cytokine* 45: 132-140.
- De Luca C, Picardo M, Breathnach A & Passi S. (1996). Lipoperoxidase activity of *Pityrosporum*: characterisation of by-products and possible role in pityriasis versicolor. *Exp Dermatol* 5: 49-56.
- Delves PJ & Roitt IM. (2000). The immune system. Second of two parts. *N Engl J Med* 343: 108-117.
- de Quadros AU, Bini D, Pereira PAT, Moroni EG & Monteiro MC. (2011). Antifungal activity of some cyclooxygenase inhibitors on *Candida albicans*: PGE₂-dependent mechanism. *Folia Microbiol* 56: 349-352.
- Deva R, Ciccoli R, Kock JLF & Nigam S. (2001). Involvement of aspirin-sensitive oxylipins in vulvovaginal candidiasis. *FEMS Microbiol Lett* 198: 37-43.
- Deva R, Ciccoli R, Schewe T, Kock JLF & Nigam S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochim Biophys Acta* 1486: 299-311.
- Deva R, Shankaranarayanan P, Ciccoli R & Nigam S. (2003). *Candida albicans* induces selectively transcriptional activation of cyclooxygenase-2 in HeLa cells: Pivotal roles of toll-like receptors, p38 mitogen-activated protein kinase, and NF-κB. *J Immunol* 171: 3047-3055.
- Dixon B. (1991). Drug discovery. Prostaglandins from yeast could lower cost. *Biotechnol* 9: 604.
- Ells R. (2008). The effect of arachidonic acid on lipid metabolism and biofilm formation of two closely related *Candida* species. Magister Scientiae dissertation, Department of Microbial, Biochemical and Food Biotechnology,

- University of the Free State, Bloemfontein, South Africa.
<http://etd.uovs.ac.za/ETD-db//theses/available/etd-10122009-095911/unrestricted/EllsR.pdf>
- Erb-Downward JR & Huffnagle GB. (2007). *Cryptococcus neoformans* produces authentic prostaglandin E₂ without a cyclooxygenase. *Eukaryot Cell* 6: 346-350.
- Erb-Downward JR, Noggle RM, Williamson PR & Huffnagle GB. (2008). The role of laccase in prostaglandin production by *Cryptococcus neoformans*. *Mol Microbiol* 68: 1428-1437.
- Erb-Downward JR & Noverr MC. (2007). Characterization of prostaglandin E₂ production by *Candida albicans*. *Infect Immun* 75: 3498-3505.
- Eschenfeldt WH, Zhang Y, Samaha H, Stols L, Eirich LD, Wilson CR & Donnelly MI. (2003). Transformation of fatty acids catalyzed by cytochrome P450 monooxygenase enzymes of *Candida tropicalis*. *Appl Environ Microbiol* 69: 5992-5999.
- Fabricius D, Neubauer M, Mandel B, Schütz C, Viardot A, Vollmer A, Jahrsdörfer B & Debatin K-M. (2010). Prostaglandin E₂ inhibits IFN- α secretion and Th1 costimulation by human plasmacytoid dendritic cells via E-prostanoid 2 and E-prostanoid 4 receptor engagement. *J Immunol* 184: 677-684.
- Fox SR, Akpınar A, Prabhune AA, Friend J & Ratledge C. (2000b). The biosynthesis of oxylipins of linoleic and arachidonic acids by the sewage fungus *Leptomitus lacteus*, including the identification of 8*R*-hydroxy-9*Z*,12*Z*-octadecadienoic acid. *Lipids* 35: 23-30.
- Fox SR, Hamberg M, Friend J & Ratledge C. (2000a). Evidence for the mitochondrial biosynthesis of 3*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid in the yeast *Dipodascopsis uninucleata*. *Lipids* 35: 1205-1214.
- Fuloria M, Eckman DM, Leach DA & Aschner JL. (2004). 20-Hydroxyeicosatetraenoic acid is a vasoconstrictor in the newborn piglet pulmonary microcirculation. *Am J Physiol Lung Cell Mol Physiol* 287: L360-L365.

- Funk CD. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294: 1871-1875.
- German JB, Gershwin ME & Berger A. (1995). Non-methylene interrupted fatty acids as immunomodulators. Patent no. 5,456,912.
- Ghosh S, Howe N, Volk K, Tati S, Nickerson KW & Petro TM. (2010). *Candida albicans* cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. *FEMS Immunol Med Microbiol* 60: 63-73.
- Goodnight SH Jr, Harris WS, Connor WE & Illingworth DR. (1982). Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. *Arterioscler Thromb Vasc Biol* 2: 87-113.
- Goodwin JS & Ceuppens J. (1983). Regulation of the immune response by prostaglandins. *J Clin Immunol* 3: 295-315.
- Groenewald EG & van der Westhuizen AJ. (1997). Prostaglandins and related substances in plants. *Bot Rev* 63: 199-220.
- Haas-Stapleton EJ, Lu Y, Hong S, Arita M, Favoreto S, Nigam S, Serhan CN & Agabian N. (2007). *Candida albicans* modulates host defense by biosynthesizing the pro-resolving mediator Resolvin E1. *PLoS One* 2: e1316.
- Hamberg M. (1986). Isolation and structures of lipoxygenase products from *Saprolegnia parasitica*. *Biochim Biophys Acta* 876: 688-692.
- Hamberg M, Herman CA & Herman RP. (1986). Novel biological transformations of 15-Ls-hydroperoxy-5,8,11,13-eicosatetraenoic acid. *Biochim Biophys Acta* 18: 447-457.
- Harizi H & Gualde N. (2006). Pivotal role of PGE₂ and IL-10 in the cross-regulation of dendritic cell-derived inflammatory mediators. *Cell Mol Immunol* 3: 271-277.
- Harizi H, Juzan M, Pitard V, Moreau JF & Gualde N. (2002). Cyclooxygenase-2-issued prostaglandin E₂ enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J Immunol* 168: 2255-2263.

- Hata AN & Breyer RM. (2004). Pharmacology and signalling of prostaglandin receptors: Multiple roles in inflammation and immune modulation. *Pharmacol Ther* 103: 147-166.
- Hatae N, Sugimoto Y & Ichikawa A. (2002). Prostaglandin receptors: advances in the study of EP3 receptor signaling. *J Biochem* 131: 781-784.
- Henderson WR. (1994). The role of leukotrienes in inflammation. *Ann Intern Med* 121: 684-697.
- Henriques M, Martins M, Azeredo J & Oliveira R. (2007). Effect of farnesol on *Candida dubliniensis* morphogenesis. *Lett Appl Microbiol* 44: 199-205.
- Herman RP & Hamberg M. (1987). Properties of the soluble arachidonic acid 15-lipoxygenase and 15-hydroperoxide isomerase from the oomycete *Saprolegnia parasitica*. *Prostaglandins* 34: 129-139.
- Herman RP & Herman CA. (1985). Prostaglandins or prostaglandin-like substances are implicated in normal growth and development in oomycetes. *Prostaglandins* 29: 819-830.
- Higashiyama K, Fujikawa S, Park EY & Shimizu S. (2002). Production of arachidonic acid by *Mortierella* fungi. *Biotechnol Bioprocess Eng* 7: 252-262.
- Hiltunen JK, Chen Z, Haapalainen AM, Wierenga RK & Kastaniotis AJ. (2010). Mitochondrial fatty acid synthesis – An adopted set of enzymes making a pathway of major importance for the cellular metabolism. *Prog Lipid Res* 49: 27-45.
- Hiltunen JK, Okubo F, Kursu KJ, Autio KJ & Kastaniotis AJ. (2005). Mitochondrial fatty acid synthesis and maintenance of respiratory competent mitochondria in yeast. *Biochem Soc Trans* 33: 1162-1165.
- Hiltunen JK, Schonauer MS, Autio KJ, Mittelmeier TM, Kastaniotis AJ & Dieckmann CL. (2009). Mitochondrial FAS type II – more than just fatty acids. *J Biol Chem* 284: 9011-9015.

- Holland HL, Brown FM, Rao JA & Chencharah PC. (1988). Synthetic approaches to the prostaglandins using microbial biotransformations. *J Ind Microbiol* 31: 191-195.
- Hornby JM, Jensen EC, Lisec AD, Tasto JJ, Jahnke B, Shoemaker R, Dussault P & Nickerson KW. (2001). Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* 67: 2982-2992.
- Hörnsten L, Su C, Osbourn AE, Garosi P, Hellman U, Wernstedt C & Oliw EH. (1999). Cloning of linoleate diol synthase reveals homology with prostaglandin H synthases. *J Biol Chem* 274: 28219-28224.
- Jahn U, Galano J-M & Durand T. (2008). Beyond prostaglandins—chemistry and biology of cyclic oxygenated metabolites formed by free-radical pathways from polyunsaturated fatty acids. *Angew Chem Int Ed* 47: 5894-5955.
- Jensen EC, Ogg C & Nickerson KW. (1992). Lipoxygenase inhibitors shift the yeast/mycelium dimorphism in *Ceratocystis ulmi*. *Appl Environ Microbiol* 58: 2505-2508.
- Kalinski P, Hilkens CMU, Snijders A, Snijdwint FCM & Kapsenberg M. (1997). IL-12-deficient dendritic cells, generated in the presence of prostaglandin E₂ promote Type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 159: 28-35.
- Kalo-Klein A & Witkin SS. (1990). Prostaglandin E₂ enhances and gamma interferon inhibits germ tube formation in *Candida albicans*. *Infect Immun* 58: 260-262.
- Kapoor R & Huang YS. (2006). Gamma linolenic acid: An anti-inflammatory omega-6 fatty acid. *Curr Pharm Biotech* 7: 531-534.
- Kebaara BW, Langford ML, Navarathna DHMLP, Dumitru R, Nickerson KW & Atkin AL. (2008). *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous-growth induction. *Eukaryot Cell* 7: 980-987.
- Kendrick A & Ratledge C. (1992). Lipids of selected molds grown for production of n-3 and n-6 polyunsaturated fatty acids. *Lipids* 27: 15-20.

- Kim D, Cryle MJ, De Voss JJ & de Montellano PRO. (2007). Functional expression and characterization of cytochrome P450 52A21 from *Candida albicans*. Arch Biochem Biophys 464: 213-220.
- Kock JLF, Coetzee DJ, van Dyk MS, Truscott M, Botha A & Augustyn OP. (1992). Evidence for, and taxonomic value of, an arachidonic acid cascade in the Lipomycetaceae. Antonie Leeuwenhoek 62: 251-259.
- Kock JLF, Coetzee DJ, van Dyk MS, Truscott M, Cloete FC, van Wyk V & Augustyn OPH. (1991). Evidence for pharmacologically active prostaglandins in yeasts. S Afr J Sci 87: 73-75.
- Kock JLF, Jansen van Vuuren D, Botha A, Van Dyk MS, Coetzee DJ, Botes PJ, Shaw N, Friend J, Ratledge C, Roberts AD & Nigam S. (1997). The production of biologically active 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE) and linoleic acid metabolites by *Dipodascopsis*. Syst Appl Microbiol 20: 39-49.
- Kock JLF, Sebolai OM, Pohl CH, van Wyk PWJ & Lodolo EJ. (2007). Oxylipin studies expose aspirin as antifungal. FEMS Yeast Res 7: 1207-1217.
- Kock JLF, Strauss CJ, Pohl CH & Nigam S. (2003). The distribution of 3-hydroxy oxylipins in fungi. Prostaglandins Other Lipid Mediat 71: 85-96.
- Kock JLF, Strauss CJ, Pretorius EE, Pohl CH, Bareetseng AS, Botes PJ, Van Wyk PWJ, Schoombie SW & Nigam S. (2004). Revealing yeast movement in confined space. S Afr J Sci 100: 237-240.
- Kock JLF, Venter P, Linke D, Schewe T & Nigam S. (1998). Biological dynamics and distribution of 3-hydroxy fatty acids in the yeast *Dipodascopsis uninucleata* as investigated by immunofluorescence microscopy. Evidence for a putative regulatory role in the sexual reproductive cycle. FEBS Lett 427: 345-348.
- Kock JLF, Venter P, Smith DP, van Wyk PWJ, Botes PJ, Coetzee DJ, Pohl CH, Botha A, Riedel K-H & Nigam S. (2000). A novel oxylipin-associated 'ghosting' phenomenon in yeast flocculation. Antonie van Leeuwenhoek 77: 401-406.

- Konturek SJ & Pawlik W. (1986). Physiology and pharmacology of prostaglandins. *Dig Dis Sci* 31: 6S-19S.
- Kruppa M, Krom BP, Chauhan N, Bambach AV, Cihlar RL & Calderone RA. (2004). The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot Cell* 3: 1062-1065.
- Kull F, Ohlson E & Haeggström JZ. (1999). Cloning and characterization of a bifunctional leukotriene A4 hydrolase from *Saccharomyces cerevisiae*. *J Biol Chem* 274: 34683-34690.
- Kull F, Ohlson E, Lind B & Haeggström JZ. (2001). *Saccharomyces cerevisiae* leukotriene A4 hydrolase: Formation of leukotriene B₄ and identification of catalytic residues. *Biochemistry* 40: 12695-12703.
- Kurtzman CP, Vesonder RF & Smiley MJ. (1974). Formation of extracellular 3-D-hydroxy palmitic acid by *Saccharomycopsis malanga*. *Mycologia* 66: 580-587.
- Lamacka M & Sajbidor J. (1995). The occurrence of prostaglandins and related compounds in lower organisms. *Prostaglandins Leukot Essent Fatty Acids* C52: 357-364.
- Lamacka M & Sajbidor J. (1998). The content of prostaglandins and their precursors in *Mortierella* and *Cunninghamella* species. *Lett Appl Microbiol* 26: 224-226.
- Lambert IA. (1994). Eicosanoids and cell volume regulation. *Cellular and Molecular Physiology of Cell Volume Regulation*. CRC Press, London, Tokyo. pp 279-298.
- Larsen TB, Gutterman DD, Sato A, Toyama K, Campbell WB, Zeldin DC, Manthathi VL, Falck JR & Miura H. (2008). Hydrogen peroxide inhibits cytochrome P450 epoxygenases interaction between two endothelium-derived hyperpolarizing factors. *Circ Res* 102: 59-67.
- Leeuw NJ, Kock JLF, Pohl CH, Bareetseng AS, Sebolai OM, Joseph M, Strauss CJ, Botes PJ, Van Wyk PWJ & Nigam S. (2006). Oxylipin covered ascospores of *Eremothecium coryli*. *Antonie Leeuwenhoek* 89: 91-97.

- Leeuw NJ, Swart CW, Ncango DM, Pohl CH, Sebolai OM, Strauss CJ, Botes PJ, van Wyk PWJ, Nigam S & Kock JLF. (2007). Acetylsalicylic acid as antifungal in *Eremothecium* and other yeasts. *Antonie van Leeuwenhoek* 91: 393-405.
- Li DC, Lui ZW & Lu J. (2001). Purification and characterization of lipoxygenase from the thermophilic fungus *Thermomyces lanuginosus*. *Mycol Res* 105: 190-194.
- Ma Y-H, Gebremedhin D, Schwartzman ML, Falck JR, Clark JE, Masters BS, Harder DR & Roman RJ. (1993). 20-Hydroxyeicosatetraenoic acid is an endogenous vasoconstrictor of canine renal arcuate arteries. *Circ Res* 72: 126-136.
- Mazur P, Meyers HV, Nakanishi K, El-Zayat AAE & Champe SP. (1990). Structural elucidation of sporogenic fatty acid metabolites from *Aspergillus nidulans*. *Tetrahedron Lett* 31: 3837-3840.
- Mazur P, Nakanishi K, El-Zayat AAE & Champe SP. (1991). Structure and synthesis of sporogenic Psi factors from *Aspergillus nidulans*. *J Chem Soc Chem Commun* 20: 1486-1487.
- McCoy JM, Wick JR & Audoly LP. (2002). The role of prostaglandin E₂ receptors in the pathogenesis of rheumatoid arthritis. *J Clin Invest* 110: 651-658.
- Milne GL, Yin H & Morrow JD. (2008). Human biochemistry of the isoprostane pathway. *J Biol Chem* 283: 15533-15537.
- Montagnoli C, Sandini S, Bacci A, Romani L & La Valle R. (2004). Immunogenicity and protective effect of recombinant enolase of *Candida albicans* in a murine model of systemic candidiasis. *Med Mycol* 42: 319-324.
- Morrow JD, Awad JA, Boss HJ, Blair IA & Roberts LJ. (1992). Non-cyclooxygenase-derived prostanoids (F₂-isoprostanes) are formed *in situ* on phospholipids. *Proc Natl Acad Sci* 89: 10721-10725.
- Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF & Roberts LJ. (1990). A series of prostaglandin F₂-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci* 87: 9383-9387.

- Mueller MJ. (2004). Archetype signals in plants: the phytoprostanes. *Curr Opin Plant Biol* 7: 441-448.
- Murakami M & Kudo I. (2004). Recent advances in molecular biology and physiology of the prostaglandin E₂-biosynthetic pathway. *Prog Lipid Res* 43: 3-35.
- Navarathna DHMLP, Hornby JM, Krishnan N, Parkhurst A, Duhamel GE & Nickerson KW. (2007b). Effect of farnesol on a mouse model of systemic candidiasis, determined by use of a DPP3 knockout mutant of *Candida albicans*. *Infect Immun* 75: 1609-1618.
- Navarathna DHMLP, Nickerson KW, Duhamel GE, Jerrels TR & Petro TM. (2007a). Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. *Infect Immun* 75: 4006-4011.
- Nazzaro-Porro M, Passi S, Picardo M, Mercantini R & Breathnach AS. (1986). Lipoxygenase activity of *Pityrosporum* *in vitro* and *in vivo*. *J Invest Dermatol* 87: 108-112.
- Ncango MD, Pohl CH, Sebolai OM, Botes PJ, Strauss CJ, Joseph M, Van Wyk PWJ, Nigam S & Kock JLF. (2006). Oxylipin-coated hat-shaped ascospores of *Ascoidea corymbosa*. *Can J Microbiol* 52: 1046-1050.
- Needleman P, Turk J, Jakschik BA, Morrison AR & Lefkowitz JB. (1986). Arachidonic acid metabolism. *Ann Rev Biochem* 55: 69-102.
- Newton RF & Roberts SM. (1997). Prostaglandins and thromboxanes. London: Butterworth.
- Nickerson KW, Atkin AL & Hornby JM. (2006). Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl Environ Microbiol* 72: 3805-3813.
- Nigam S, Ciccoli R, Ivanov I, Szczepanski M & Deva R. (2011). On mechanism of quorum sensing in *Candida albicans* by 3(*R*)-hydroxy-tetradecaenoic acid. *Curr Microbiol* 62: 55-63.
- Nigam S, Kumar SG & Kock JLF. (1996). Biological effects of 3-HETE, a novel compound of the yeast *Dipodascopsis uninucleata*, on mammalian cells. 10th

- International Conference on Prostaglandins and Related Compounds. Vienna, Austria 39, Sept.
- Nigam S, Schewe T & Kock JLF. (1999). 3(*R*)-Hydroxy oxylipins-a novel family of oxygenated polyenoic fatty acids of fungal origin. *Adv Exp Med Biol* 469: 663-668.
- Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC & Liao JK. (1999). Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* 20: 1276-1279.
- Noverr MC, Erb-Downward JR & Huffnagle GB. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev* 16: 517-533.
- Noverr MC & Huffnagle GB. (2004). Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect Immun* 72: 6206-6210.
- Noverr MC, Phare SM, Toews GB, Coffey MJ & Huffnagle GB. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* 69: 2957-2963.
- Noverr MC, Toews GB & Huffnagle GB. (2002). Production of prostaglandins and leukotrienes by pathogenic fungi. *Infect Immun* 70: 400-402.
- Oh KB, Miyazawa H, Naito T & Matsuoka H. (2001). Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *P Natl Acad Sci* 98: 4664-4668.
- Osterholzer JJ, Surana R, Milam JE, Montano GT, Chen GH, Sonstein J, Curtis JL, Huffnagle GB, Toews GB & Olszewski MA. (2009). Cryptococcal urease promotes the accumulation of immature dendritic cells and a non-protective T2 immune response within the lung. *Am J Pathol* 174: 932-943.
- Park J, Lee S, Choi J, Ahn K, Park B, Park J, Kang S & Lee Y-H. (2008). Fungal cytochrome P450 database. *BMC Genomics* 9: 402.

- Pelletier S, Dubé J, Villeneuve A, Gobeil F Jr, Yang Q, Battistini B, Guillemette G & Sirois P. (2001). Prostaglandin E₂ increases cyclic AMP and inhibits endothelin-1 production/secretion by guinea-pig tracheal epithelial cells through EP4 receptors. *Br J Pharmacol* 132: 999-1008.
- Perraud X & Kermasha S. (2000). Characterization of lipoxygenase extracts from *Penicillium* spp. *JAOCS* 77: 335-342.
- Perraud X, Kermasha S & Bisakowski B. (1999). Characterization of a lipoxygenase extract from *Geotrichum candidum*. *Process Biochem* 34: 819-827.
- Plotkin LI, Mathov I, Squiquera L & Leoni J. (1998). Arachidonic acid released from epithelial cells by *Malassezia furfur* phospholipase A₂: a potential pathophysiologic mechanism. *Mycologia* 90: 163-169.
- Pohl CH, Botha A, Kock JLF, Coetzee DJ, Botes PJ, Schewe T & Nigam S. (1998). Oxylipin formation in fungi: biotransformation of arachidonic acid to 3-hydroxy-5,8-tetradecadienoic acid by *Mucor genevensis*. *Biochem Biophys Res Commun* 253: 703-706.
- Prabhune A, Fox SR & Ratledge C. (2002). Transformation of arachidonic acid to 19-hydroxy- and 20-hydroxy-eicosatetraenoic acids using *Candida bombicola*. *Biotechnol Lett* 24: 1041-1044.
- Profita M, Sala A, Bonanno A, Riccobono L, Siena L, Melis MR, Giorgi RD, Mirabella F, Gjomarkaj M, Bonsignore G & Vignola AM. (2003). Increased prostaglandin E₂ concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J Allergy Clin Immunol* 112: 709-716.
- Qiao M-F, Ji N-Y, Miao F-P & Yin X-L. (2011). Steroids and an oxylipin from an algicolous isolate of *Aspergillus flavus*. *Magn Reson Chem* 49: 366-369.
- Rajakariar R, Yaqoob MM & Gilroy DW. (2006). COX-2 in inflammation and resolution. *Pharmacology* 6: 199-207.
- Ramage G, Saville SP, Thomas DP & López-Ribot JL. (2005). *Candida* biofilms: an update. *Eukaryot Cell* 4: 633-638.

- Ramage G, Saville SP, Wickes BL & Lopez-Ribot JL. (2002). Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl Environ Microbiol* 68: 5459-5463.
- Ramage G, Vande Walle K, Wickes BL & Lopez-Ribot JL. (2001a). Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 45: 2475-2479.
- Ramage G, Vande Walle K, Wickes BL & Lopez-Ribot JL. (2001b). Biofilm formation by *C. dubliniensis*. *J Clin Microbiol* 39: 3234-3240.
- Roberts LJ & Milne LG. (2009). Isoprostanes. *J Lipid Res* 50: S219-S223.
- Rocca B & FitzGerald GA. (2002). Cyclooxygenases and prostaglandins: shaping up the immune response. *Int Immunopharmacol* 2: 603-630.
- Romagnoli G, Nisini R, Chiani P, Mariotti S, Teloni R, Cassone A & Torosantucci A. (2004). The interaction of human dendritic cells with yeast and germ-tube forms of *Candida albicans* leads to efficient fungal processing, dendritic cell maturation, and acquisition of a Th1 response-promoting function. *J Leukoc Biol* 75: 117-126.
- Romani L. (2000). Innate and adaptive immunity in *Candida albicans* infections and saprophytism. *J Leukoc Biol* 68: 175-179.
- Rooney PJ & Klein BS. (2002). Linking fungal morphogenesis with virulence. *Cell Microbiol* 4: 127-137.
- Samuelsson B, Dahlén SE, Lindgren JA, Rouzer CA & Serhan CN. (1987). Leukotrienes and Lipoxins: Structures, biosynthesis and biological effects. *Science* 237: 1171-1176.
- Santoni G, Boccanera M, Adriani D, Lucciarini R, Amantini C, Morrone S, Cassone A & De Bernardis F. (2002). Immune cell-mediated protection against vaginal candidiasis: Evidence for a major role of vaginal CD4⁺ T cells and possible participation of other local lymphocyte effectors. *Infect Immun* 70: 4791-4797.

- Sato T, Watanabe T, Mikami T & Matsumoto T. (2004). Farnesol, a morphogenetic autoregulatory substance in the dimorphic fungus *Candida albicans*, inhibits hyphae growth through suppression of a mitogen-activated protein kinase cascade. *Biol Pharm Bull* 27: 751-752.
- Satoh T, Matzuda Y, Takashio M, Satoh K, Beppu T & Arima K. (1976). Isolation of lipoxygenase-like enzyme from *Fusarium oxysporum*. *Agricult Biologic Chem* 40: 953-961.
- Schaller M, Mailhammer R & Korting HC. (2002). Cytokine expression induced by *Candida albicans* in a model of cutaneous candidosis based on reconstituted human epidermis. *J Med Microbiol* 51: 672-676.
- Sebolai OM, Kock JLF, Pohl CH, Botes PJ & Nigam S. (2004). Report on the discovery of a novel 3-hydroxy oxylipin cascade in the yeast *Saccharomycopsis synnaedendra*. *Prostaglandins Other Lipid Mediat* 74: 139-146.
- Sebolai OM, Kock JLF, Pohl CH, Botes PJ, Strauss CJ, Van Wyk PWJ & Nigam S. (2005). The presence of 3-hydroxy oxylipins on the ascospore surfaces of some species representing *Saccharomycopsis* Schionning. *Can J Microbiol* 51: 605-612.
- Sebolai OM, Kock JLF, Pohl CH, Smith DP, Botes PJ, Pretorius EE, van Wyk PWJ & Nigam S. (2001). Bioprospecting for novel hydroxyoxylipins in fungi: presence of 3-hydroxy palmitic acid in *Saccharomycopsis malanga*. *Antonie van Leeuwenhoek* 80: 311-315.
- Sebolai OM, Pohl CH, Botes PJ, Strauss CJ, Van Wyk PWJ, Botha A & Kock JLF. (2007). 3-Hydroxy fatty acids found in capsules of *Cryptococcus neoformans*. *Can J Microbiol* 53: 809-812.
- Segal E. (2005). *Candida*, still number one – what do we know and where are we going from there? *Mycoses* 48: 3-11.

- Semighini CP, Hornby JM, Dumitru R, Nickerson KW & Harris SD. (2006). Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. *Mol Microbiol* 59: 753-764.
- Serhan CN, Arita M, Hong S & Gotlinger K. (2004). Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their endogenous aspirin-triggered epimers. *Lipids* 39: 1125-1132.
- Serhan CN, Chiang N & Van Dyke TE. (2008b). Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 8: 349-361.
- Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G & Moussignac RL. (2002). Resolvins: A family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med* 196: 1025-1037.
- Serhan CN, Yacoubian S & Yang R. (2008a). Anti-inflammatory and pro-resolving lipid mediators. *Annu Rev Pathol* 3: 279-312.
- Shechter G & Grossman S. (1983). Lipoxygenase from baker's yeast: Purification and properties. *Int J Biochem* 15: 1295-1304.
- Shibata Y, Henriksen RA, Honda I, Nakamura RM & Myrvik QN. (2005). Splenic PGE₂-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol* 78: 1281-1290.
- Shiraki Y, Ishibashi Y, Hiruma M, Nishikawa A & Ikeda S. (2008). *Candida albicans* abrogates the expression of interferon- γ -inducible protein-10 in human keratinocytes. *FEMS Immunol Med Microbiol* 54: 122-128.
- Shoun H, Sudo Y, Yasuo Seto Y & Beppu T. (1983). Purification and properties of a cytochrome P-450 of a fungus, *Fusarium oxysporum*. *J Biochem* 94: 1219-1229.
- Sigal E. (1991). The molecular biology of mammalian arachidonic acid metabolism. *Am J Physiol* 260: 13-18.

- Sih CJ, Ambrus G, Foss P & Lai CJ. (1969). A general biochemical synthesis of oxygenated prostaglandins E. *J Am Chem Soc* 91: 3685-3687.
- Smeekens SP, van de Veerdonk FL, van der Meer JWM, Kullberg BJ, Joosten LAB & Netea MG. (2010). The *Candida* Th17 response is dependent on mannan- and β -glucan-induced prostaglandin E₂. *Int Immunol* 22: 889-895.
- Smith DA, Nicholls S, Morgan BA, Brown AJP & Quinn J. (2004). A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol Biol Cell* 15: 4179-4190.
- Smith DP, Kock JLF, Van Wyk PWJ, Venter P, Coetzee DJ, Van Heerden E, Linke D & Nigam S. (2000). The occurrence of 3-hydroxy oxylipins in the ascomycetous yeast family Lipomycetaceae. *S Afr J Sci* 96: 247-249.
- Smith WL. (1989). The eicosanoids and their biochemical mechanism of action. *Biochem J* 259: 315-324.
- Smith WL, Garavito RM & DeWitt DL. (1996). Prostaglandin endoperoxide H synthases (cyclooxygenase)-1 and -2. *J Biol Chem* 271: 33157-33160.
- Sornasse T, Larenas PV, Davis KA, deVries JE & Yssel H. (1996). Differentiation and stability of T Helper 1 and 2 cells derived from naive human neonatal CD4⁺ T cells, analyzed at the single-cell level. *J Exp Med* 184: 473-483.
- Strauss CJ, Kock JLF, van Wyk PWJ, Lodolo EJ, Pohl CH & Botes PJ. (2005). Bioactive oxylipins in *Saccharomyces cerevisiae*. *J Inst Brew* 111: 304-308.
- Strauss CJ, Van Wyk PWJ, Lodolo EJ, Botes PJ, Pohl CH, Nigam S & Kock JLF. (2006). Oxylipins associated co-flocculation in yeasts. *J Inst Brew* 111: 66-71.
- Strong R, Miller RA, Astle CM, Floyd RA, Flurkey K, Hensley KL, Javors MA, Leeuwenburgh C, Nelson JF, Ongini E, Nadon NL, Warner HR & Harrison DE. (2008). Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice. *Aging Cell* 7: 641-650.

- Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE & Coleman DC. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV infected individuals. *Microbiology* 141: 1507-1521.
- Su C & Oliw EH. (1998). Manganese lipoxygenase. Purification and characterization. *J Biol Chem* 273: 13072-13079.
- Swart CW, van Wyk PWJ, Pohl CH, Kriel WM & Kock JLF. (2011). The influence of mitochondrial inhibitors on the life cycle of *Phytophthora*. *Afr J Microbiol Res* 5: 3175-3180.
- Tanaka T, Morishige J, Takimoto T, Takai Y & Satouchi K. (2001). Metabolic characterization of sciadonic acid as an effective substitute for arachidonate of phosphatidylinositol. *Eur J Biochem* 268: 4928-4939.
- Traynor TR, Kuziel WA, Toews GB & Huffnagle GB. (2000). CCR2 expression determines T1 versus T2 polarization during pulmonary *Cryptococcus neoformans* infection. *J Immunol* 164: 2021-2027.
- Tsitsigiannis DI, Bok JW, Andes D, Nielsen KF, Frisvad JC & Keller NP. (2005b). *Aspergillus* cyclooxygenase-like enzymes are associated with prostaglandin production and virulence. *Infect Immun* 73: 4548-4559.
- Tsitsigiannis DI & Keller NP. (2007). Oxylipins as developmental and host-fungal communication signals. *Trends Microbiol* 15: 109-118.
- Tsitsigiannis DI, Kowieski TM, Zarnowski R & Keller NP. (2004). Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. *Eukaryot Cell* 3: 1398-1411.
- Tsitsigiannis DI, Kowieski TM, Zarnowski R & Keller NP. (2005a). Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. *Microbiology* 151: 1809-1821.

- Tsukada K, Takahashi K & Nebeta K. (2010). Biosynthesis of jasmonic acid in a plant pathogenic fungus, *Lasiodiplodia theobromae*. *Phytochemistry* 71: 2019-2023.
- Van Bogaert INA, Groeneboer S, Saerens K & Soetaert W. (2011). The role of cytochrome P450 monooxygenases in microbial fatty acid metabolism. *FEBS J* 278: 206-221.
- van der Meer JWM, van de Veerdonk FL, Joosten LAB, Kullberg BJ & Netea MG. (2010). Severe *Candida* spp. infections: new insights into natural immunity. *Int J Antimicrob Ag* S36: S58-S62.
- van der Pouw Kraan TCTM, Boeije LCM, Smeenk RJT, Wijdenes J & Aarden LA. (1995). Prostaglandin-E₂ is a potent inhibitor of human interleukin-12 production. *J Exp Med* 181: 775-779.
- Van Dyk MS, Kock JLF, Coetzee DJ, Augustyn OPH & Nigam S. (1991). Isolation of a novel arachidonic acid metabolite 3-hydroxy-5,8,11,14-eicosatetraenoic acid (3-HETE) from the yeast *Dipodascopsis uninucleata*. *FEBS Lett* 283: 195-198.
- Van Heerden A, Kock JLF, Botes PJ, Pohl CH, Strauss CJ, Van Wyk PWJ & Nigam S. (2005). Ascospore release from bottleshaped asci in *Dipodascus albidus*. *FEMS Yeast Res* 5: 1185-1190.
- Van Heerden A, Van Wyk PWJ, Botes PJ, Pohl CH, Strauss CJ, Nigam S & Kock JLF. (2007). The release of elongated, sheathed ascospores from bottle-shaped asci in *Dipodascus geniculatus*. *FEMS Yeast Res* 7: 173-179.
- Venter P, Kock JLF, Kumar GS, Botha A, Coetzee DJ, Botes PJ, Bhatt RK, Falck JR, Schewe T & Nigam S. (1997). Production of 3R-hydroxy-polyenoic fatty acids by the yeast *Dipodascopsis uninucleata*. *Lipids* 32: 1277-1283.
- Verstrepen KJ, Derdelinckx G, Veractert H & Delvaux FR. (2003). Yeast flocculation: what brewers should know. *Appl Microbiol Biotechnol* 61: 197-206.
- Vesonder RF, Wickerham LJ & Rohwedder WK. (1968). 3-D-Hydroxypalmitic acid: a metabolic product of the yeast NRRL Y-6954. *Can J Chem* 46: 2628-2629.

- Weaver CT, Hatton RD, Mangan PR & Harrington LE. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25: 821-852.
- Westwater C, Balish E & Schofield DA. (2005). *Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance. *Eukaryot Cell* 4: 1654-1661.
- Witkowski A, Joshi AK & Smith S. (2007). Coupling of the *de novo* fatty acid biosynthesis and lipoylation pathways in mammalian mitochondria. *J Biol Chem* 282: 14178-14185.
- Yilmaz E. (2001). Biotechnological production of prostaglandin. *Biotech Adv* 19: 387-397.
- Zeldin DC. (2001). Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem* 276: 36059-36062.
- Zhu Y, Schieber EB, McGiff JC & Balazy M. (1995). Identification of arachidonate P-450 metabolites in human platelet phospholipids. *Hypertension* 25: 854-859.

CHAPTER 2

Prostaglandin production by Candida biofilms

A part of this chapter has been published in *Medical Microbiology and Immunology* 200: 23-28, (2011).

2.1. Abstract

Arachidonic acid (AA) [20:4(n-6)] is released from infected host cells during *Candida albicans* infection and may serve as carbon source for yeast growth and as precursor for the production of biologically active eicosanoids, such as prostaglandin E₂ (PGE₂) by *C. albicans*. Prostaglandin E₂ is an important virulence factor in *C. albicans* and involved in biofilm formation. Biofilms increase damage in host cells and are more resistant to antifungal drugs than planktonic yeast cells. This study evaluated the production of prostaglandins, PGE₂ and PGF_{2α}, by biofilms of *C. albicans* and the closely related *C. dubliniensis*. Since, the mechanism involved in this production is still unclear, it was of interest to investigate the effect of different AA metabolism inhibitors on PGE₂ production by biofilms of these *Candida* species. The experiments were done by growing *Candida* biofilms in the presence of AA as well as cytochrome P450 (CYP450), multicopper oxidase, cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors. The PGE₂ and PGF_{2α} concentrations were determined by a monoclonal enzyme-linked immunosorbent assay (ELISA) and verified with LCMS/MS. The results obtained indicate the ability of *C. albicans* and *C. dubliniensis* biofilms to produce PGE₂ and PGF_{2α}, from exogenous AA. The use of different inhibitors suggested that CYP450s and multicopper oxidases are involved in PGE₂ production by these *Candida* biofilms.

2.2. Introduction

Candida albicans and *C. dubliniensis* are closely related dimorphic yeast pathogens capable of forming biofilms (Ramage et al., 2001a, b; Sullivan et al., 1995). *Candida albicans* infections are associated with the release of the bioactive molecule, arachidonic acid (AA) [20:4(n-6)], from the infected host cell membrane (Brash, 2001; Deva et al., 2001). The released AA can be used as a carbon source by *C. albicans* and as a precursor for the synthesis of yeast eicosanoids such as prostaglandin E₂ (PGE₂) by *C. albicans* (Deva et al., 2000; Noverr et al., 2003). Eicosanoids are involved in *C. albicans* infection, affecting the host's immune responses, enhancing vascular permeability and facilitating the invasion of the host tissue/cells, as well as enhancing germ tube formation (Deva et al., 2001; Noverr et al., 2001; Noverr & Huffnagle, 2004). The production of eicosanoids by *C. dubliniensis* has not been studied, although its close relationship to *C. albicans* (Sullivan et al., 1995) might point to similar ability to produce eicosanoids.

The mechanisms involved in the production of PGE₂ by *C. albicans* are still not clear. It was speculated that cyclooxygenase-like enzymes (COX) are responsible, but this could not be confirmed through the use of different COX inhibitors as well as with BLAST analysis to search the genome of *C. albicans* for homologues of biosynthetic enzymes involved in mammalian eicosanoid production (Erb-Downward & Noverr, 2007; Noverr et al., 2001). Erb-Downward and Noverr (2007) identified a fatty acid desaturase homologue (Ole2p) and a multicopper oxidase homologue (Fet3p) to be essential enzymes involved in *C. albicans* prostaglandin production. Recently, Erb-Downward and co-workers (2008) identified the production of prostaglandins in *Cryptococcus neoformans*, and indicated that laccase, a multicopper oxidase, plays an important role in this production. This suggests that multicopper oxidases might play a significant role in eicosanoid production by pathogenic yeasts. However, this is not the only enzyme involved and questions still need to be answered regarding the enzymes upstream of the multicopper oxidase. In mammalian cells it is known that lipoxygenase enzymes (LOX) and cytochrome P450 enzymes (CYP450) play important roles in AA metabolism (Brash, 2001; Imig et al., 1999; Su et al., 1998; Wang et al., 1997). Although no LOX homologues were found in the genome of *C. albicans*, the genome

does contain at least 12 CYP450s, suggesting that some of these might be involved in AA metabolism (Erb-Downward & Noverr, 2007; Haas-Stapleton et al., 2007; Park et al., 2008).

Therefore, the objective of this study was to evaluate extracellular prostaglandin production (PGE₂ and PGF_{2α}) by biofilms of *C. albicans* and *C. dubliniensis* from exogenous AA. The production of PGE₂ by biofilms of *C. albicans* and *C. dubliniensis*, in the presence of CYP450s, multicopper oxidases, COX and LOX inhibitors was also evaluated.

2.3. Materials and methods

2.3.1. Strains used

The following strains were used in this study: *Candida albicans* NRRL Y-27077 (isolated from a skin lesion, Germany) and *C. dubliniensis* NRRL Y-17841T (isolated from oral cavity of HIV-infected patient, Dublin, Ireland). Both strains were obtained from the Agricultural Research Service Culture Collection of the United States Department of Agriculture. All strains were maintained on Yeast Malt Extract (YM) agar (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 16 g/L agar) at room temperature.

2.3.2. Biofilm formation

Strains from 24 h old cultures on YM agar plates were inoculated into 20 mL synthetic media (6.7 g/L Yeast Nitrogen Base, 10 g/L glucose) in 50 mL Falcon Polypropylene Conical centrifuge tubes (Becton Dickinson Labware, USA) and incubated at 30°C for 48 h. The cells were harvested by centrifugation (10 min at 4412 x g) (Heraeus, Megafuge 1R), washed twice with phosphate buffered saline (PBS) (OXOID, UK) and resuspended into filter sterilized RPMI-1640 medium (Sigma-Aldrich, USA). The cells were counted and diluted to 1 x 10⁶ cells/mL in 50 mL RPMI-1640 medium containing a final concentration of 500 μM AA (Sigma Aldrich, USA) (in 0.38% v/v ethanol) (Sigma Aldrich, USA). Appropriate controls

were included. These suspensions were dispensed into 90 mm polystyrene Petri dishes (Merck, RSA) and incubated at 37°C for up to 48 h, to allow biofilm formation (Ramage et al., 2001a).

2.3.3. Determination of extracellular prostaglandin concentration by ELISA

The supernatants from the biofilms were harvested, centrifuged (10 min at 4412 x g) and filtered through 0.2 µm cellulose acetate syringe filters (GEMA Medical SL, Spain). The supernatants for PGE₂ determination were then purified by the use of a PGE₂ affinity column according to the manufacturer's instructions (Cayman Chemicals, USA). The eluates were dried and resuspended in enzyme immuno assay buffer, and the PGE₂ concentrations determined after 8 h and 48 h using a monoclonal PGE₂ enzyme-linked immunosorbent assay (ELISA) (Cayman Chemicals, USA) according to the manufacturer's instructions. Additionally, the concentration PGF_{2α} was determined after 48 h using a monoclonal PGF_{2α} enzyme-linked immunosorbent assay (Cayman Chemicals) without purification of the supernatants. These ELISA kits are highly sensitive and detect as little as 15 pg/mL of PGE₂ and 9 pg/mL of PGF_{2α}. Controls included biofilms grown without AA in RPMI-1640 medium as well as RPMI medium with and without AA. Background levels of PGE₂ or PGF_{2α} detected in medium with AA alone were subtracted from the experimental samples. The experiment was done in duplicate, with each sample assayed at two dilutions and each dilution assayed in triplicate.

2.3.4. Mass spectrometry

The purified samples from above were further analyzed to verify PGE₂ production. The analyses were carried out on an ABSciex API3200QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Toronto, Canada) with an Agilent 1200 SL series HPLC front end. The samples (10 µL) were injected at a flow rate of 0.5 mL/min and the analyses were performed at 30°C. A Zorbax E clipse XDB C18, 50 mm x 4.6 mm column (Agilent Technologies, Waldbronn, Germany) was used for sample separation. The mobile phases consisted of 10 mM ammonium

formate aqueous solution in 5% methanol (solvent A) and 10 mM ammonium formate aqueous solution in 95% methanol (solvent B), the programmed elution gradient was: 0 - 5 min: 50% B, 5 - 10 min: 50 - 95% B, followed by an equilibration step for a total chromatographic run of 20 minutes. Atmospheric pressure electrospray ionization was carried out in the negative mode. Prostaglandin E₂ reference standard (Cayman Chemicals, USA) was infused into the instrument and the compound optimization feature included in Analyst™ software was used to build a 5 transition (one precursor producing five unique fragments) multiple reaction monitoring (MRM) method prior to sample separation. In an MRM method a triple quadrupole mass spectrometer allows only the mass of the ionized analyte of interest (i.e. PGE₂ with m/z of 351.2) from Q1 into the collision cell where it is fragmented and the presence and intensity of specific fragment ions unique to the precursor of interest is subsequently monitored in Q3. The ion spray voltage was 4500 V, the source temperature was 500°C, the declustering potential was 20 V and the collision energy ranged from 14–30 eV for the various fragment ions. The following 5 transitions were selected for the final LCMS/MS method: 351.2/315.2; 351.2/271.2; 351.2/333.3; 351.2/189.0 and 351.2/235.1. Only if all 5 transitions were recorded at the same retention time would the presence of PGE₂ be confirmed.

2.3.5. Germ tube assay

A small portion of a pure colony of 18-24 h old cultures on YM agar plates were inoculated in filter sterilized RPMI-1640 medium. To this suspension, purified PGE₂ from *C. albicans* and *C. dubliniensis*, as well as commercial PGE₂ (Cayman Chemicals, USA) were added to give a final PGE₂ concentration of 120 pg/mL. The tubes were incubated aerobically at 37°C for 4 h. Samples were removed and 400 cells were counted at 400x magnification using a Zeiss Axioplan light microscope. Appearance of small filaments projecting from the cell surface confirms germ tube formation (Elmer et al., 1992).

2.3.6. Determination of biomass and cell viability

The effect of growth in the presence of 500 μM AA as well as of the inhibitors, at a final concentration of 100 μM , on biofilm biomass was evaluated by scraping off the biofilms and filtering through pre-weighed 0.2 μm filters (Sartorius Stedim Biotech, Germany). The filters were dried to constant weight for 48 h at 37°C and the biomass determined. The effect of AA and inhibitors on cell viability of the biofilms was studied using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma Chemicals, USA) as described previously (Bachmann et al., 2002).

2.3.7. Inhibition of extracellular PGE_2 production

Biofilms were grown as before in the presence of 500 μM AA. Different inhibitors, diluted to a final concentration of 100 μM , in RPMI-1640 medium, were added together with AA. The inhibitors used were purchased from Sigma Aldrich, RSA. Stock solutions of 6-(2-propargyloxyphenyl)hexanoic acid (PPOH), 1-aminobenzotriazole (ABT), acetylsalicylic acid (ASA) and nordihydroguaiaretic acid (NDGA) was prepared in ethanol (0.4% v/v) and stock solutions of ammonium tetrathiomolybdate (ATM) and sodium azide was prepared in distilled water. Prostaglandin E_2 concentration was determined as described above by an ELISA assay. The experiment was done in duplicate, with each sample assayed at two dilutions and each dilution assayed in triplicate.

2.3.8. Statistical analysis

All experiments were performed in triplicate unless stated otherwise. The *t*-test was performed to determine the significance of the data sets.

2.4. Results and discussion

2.4.1. Prostaglandin production by *Candida* biofilms

Candida albicans and *C. dubliniensis* biofilms both produced PGE₂ from exogenous AA (Figure 1). It was found that *C. albicans* biofilms secrete significantly more PGE₂ than *C. dubliniensis* biofilms on the basis of biofilm biomass after 8 h and 48 h. This might also contribute to the increased virulence of *C. albicans* compared to *C. dubliniensis* as PGE₂ is known to play a role in virulence. Biofilms formed by *C. albicans* consisted predominantly out of yeast cells and biofilms by *C. dubliniensis* out of yeast and pseudohyphae. This suggests that the morphology does not contribute to the observed difference. Alem and Douglas (2005) found that *C. albicans* biofilms secreted prostaglandins *de novo* (circa 100 pg/mg). We observed similar results in the absence of AA, with *C. albicans* and *C. dubliniensis* biofilms producing 62 pg/mg (\pm 5.9) and 30 pg/mg (\pm 5.8) PGE₂, respectively. The production of PGE₂ in both these strains *de novo* and from exogenous AA were confirmed by LCMS/MS (Figure 2). This is the first report of PGE₂ production by *C. dubliniensis*. The seeming disparity in the amounts of PGE₂ detected between ELISA and LCMS/MS results can be explained by the fact that ELISA results were normalized against biomass, while the LCMS/MS results were a qualitative and not quantitative indication of PGE₂ in the supernatant.

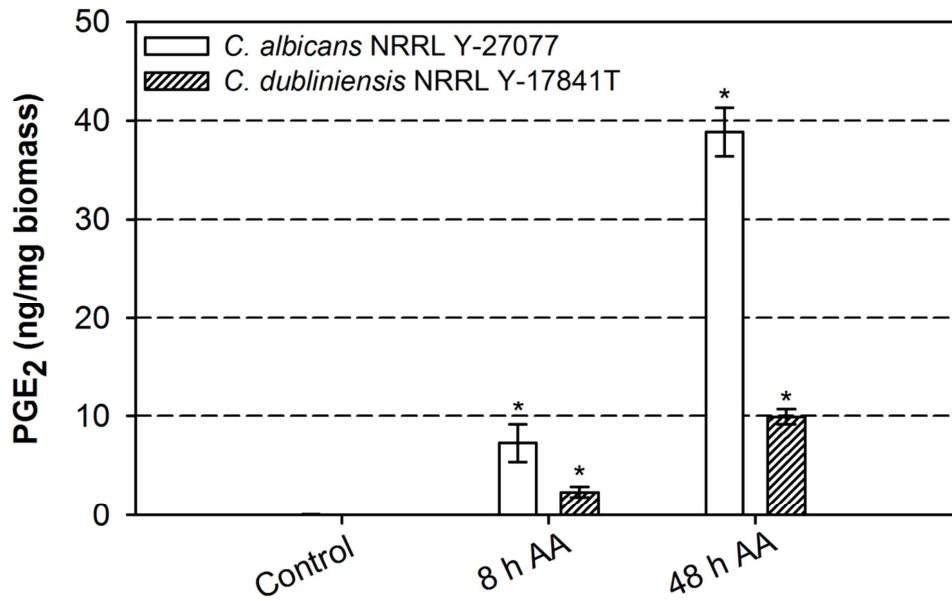


Figure 1. Prostaglandin E₂ production by *Candida albicans* and *C. dubliniensis* biofilms. Biofilms were grown for 8 and 48 h at 37°C in the absence and presence of 500 μM arachidonic acid (AA) and PGE₂ production determined by the use of a monoclonal PGE₂ EIA kit (Cayman Chemicals). Values are the mean of duplicate experiments with each sample assayed at two dilutions and each dilution assayed in triplicate and the standard deviation indicated. * Significantly different from control ($P \leq 0.01$).

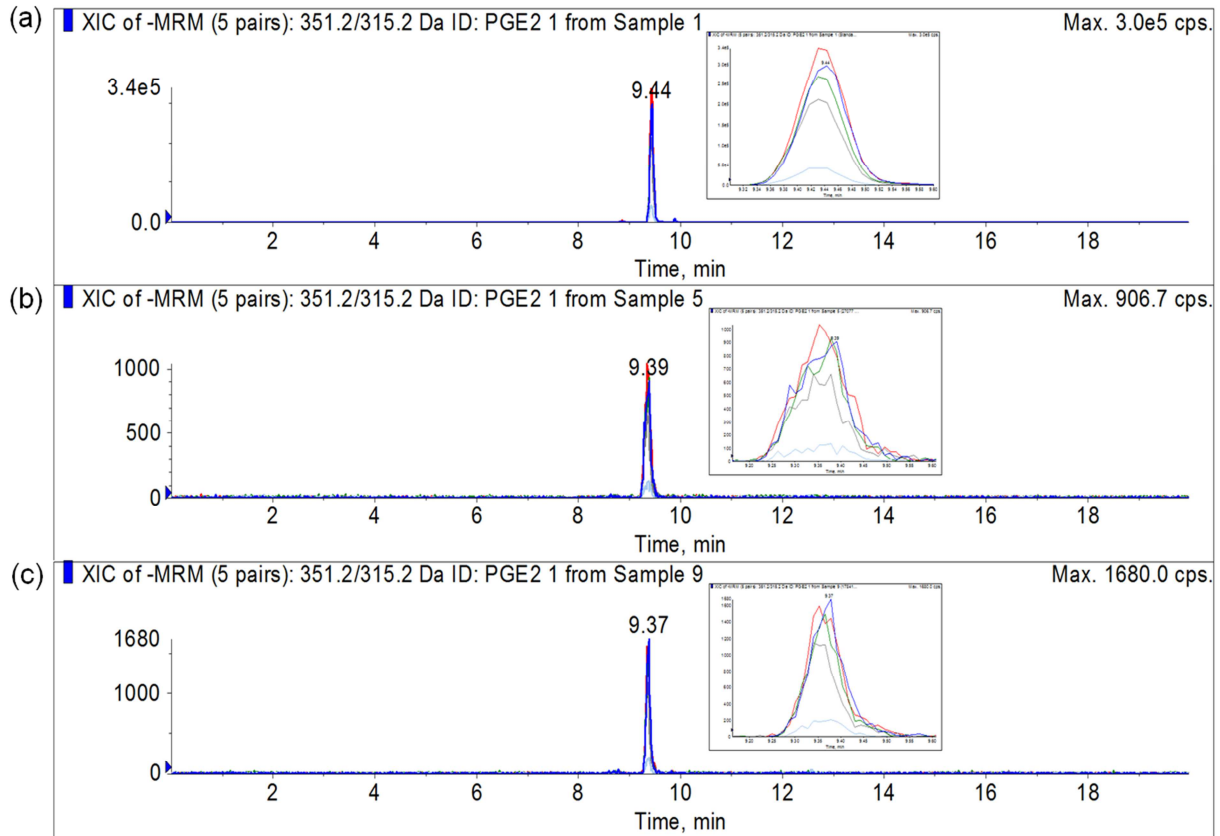


Figure 2. Chromatograms showing the five transition multiple reaction monitoring (MRM) for each of the three samples. (a) PGE₂ standard. (b) Purified PGE₂ from *C. albicans* grown in the presence of 500 μ M arachidonic acid (AA). (c) Purified PGE₂ from *C. dubliniensis* grown in the presence of 500 μ M AA. The smaller inserts show the enlarged peak with relevant transitions.

■ 351.2/315.2; ■ 351.2/271.2; ■ 351.2/333.3; ■ 351.2/189.0; ■ 351.2/235.1

It was also found that both *C. albicans* and *C. dubliniensis* are capable of producing PGF_{2 α} [0.51 ng/mg (\pm 0.13) and 0.87 ng/mg (\pm 0.12), respectively] from exogenous AA, without any PGF_{2 α} being produced in the absence of AA (Figure 3). Interestingly, *C. dubliniensis* produced more PGF_{2 α} compared to *C. albicans*. We found lower concentrations being produced compared to what has been reported for lysates of planktonic *Crypt. neoformans* (circa 8.75 ng/ml PGF_{2 α}) (Erb-Downward et al., 2008), and we also found that the biofilms of these *Candida* species produced more PGE₂ than PGF_{2 α} , whereas *Crypt. neoformans* produced more PGF_{2 α} .

However, the production of $\text{PGF}_{2\alpha}$ by these *Candida* biofilms could not be confirmed with LCMS/MS.

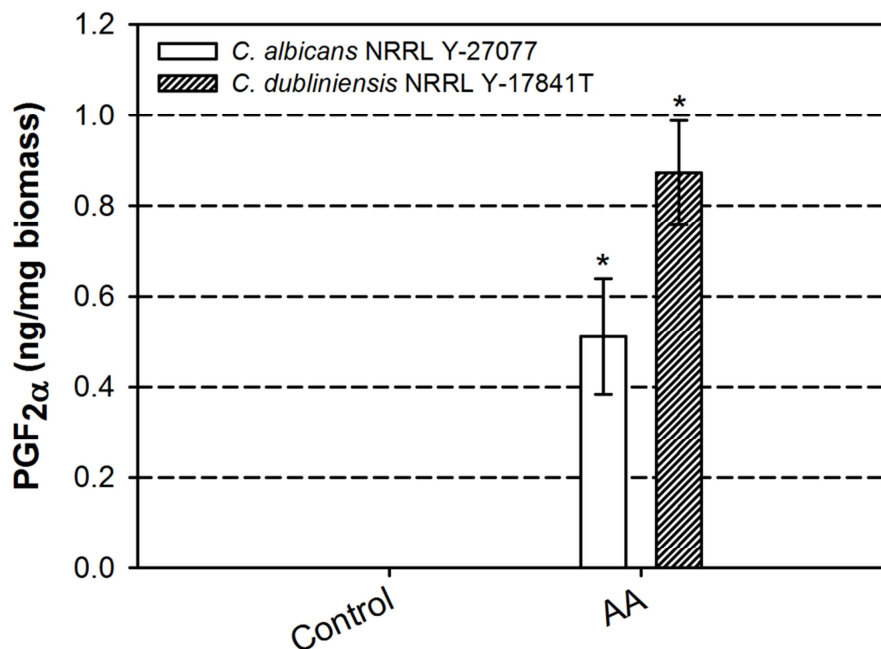


Figure 3. Prostaglandin $\text{F}_{2\alpha}$ production by *Candida albicans* and *C. dubliniensis* biofilms. Biofilms were grown for 48 h at 37°C in the absence and presence of 500 μM arachidonic acid (AA) and $\text{PGF}_{2\alpha}$ production determined by the use of a monoclonal $\text{PGF}_{2\alpha}$ EIA kit (Cayman Chemicals). Values are the mean of duplicate experiments with each sample assayed at two dilutions and each dilution assayed in triplicate and the standard deviation indicated. * Significantly different from control ($P \leq 0.01$).

2.4.2. Effect of fungal PGE_2 on germ tube formation by the *Candida* species

We evaluated the effect of purified fungal PGE_2 on germ tube production by *C. albicans* and *C. dubliniensis*. We found that PGE_2 from both species, significantly enhanced germ tube production by *C. albicans* and *C. dubliniensis*, similar to commercial PGE_2 (Figure 4). These results correlate with that found by other authors for *C. albicans* (Noverr et al., 2001; Noverr & Huffnagle, 2004). This suggests that PGE_2 might play a similar role in virulence in both *C. albicans* and *C. dubliniensis*.

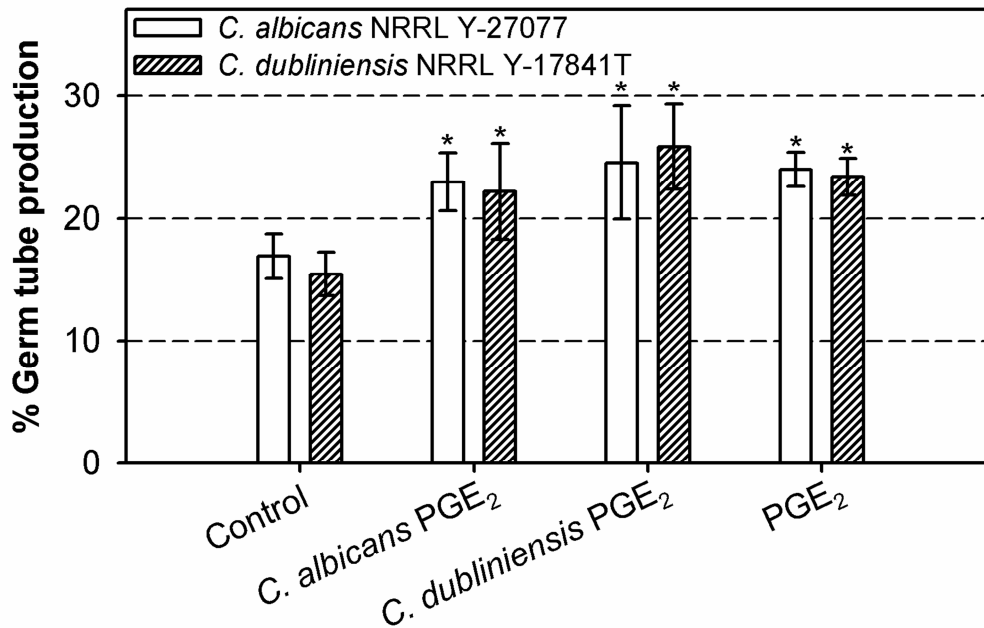


Figure 4. Effect of PGE₂ on germ tube production by *Candida albicans* and *C. dubliniensis*. Purified fungal PGE₂ or commercial PGE₂ were added at 120 pg/mL to *C. albicans* and *C. dubliniensis* in filter sterilized RPMI-1640 medium and incubated for 4 h aerobically at 37°C. The percentage germ tubes produced were determined by counting 400 cells at 400x magnification using a light microscope. * Significantly different from control ($P \leq 0.05$).

2.4.3. Effect of inhibitors on PGE₂ production by the *Candida* biofilms

Literature suggested that multicopper oxidases are involved in PGE₂ production by pathogenic yeasts (Erb-Downward et al., 2008; Erb-Downward & Noverr, 2007; Noverr et al., 2001). Therefore, we used ammonium tetrathiomolybdate (ATM) and sodium azide as multicopper oxidase inhibitors (Johannes & Majcherczyk, 2000). These inhibitors irreversibly bind to the copper atoms in the active sites of these enzymes and inactivate them. Interestingly, ATM significantly inhibited PGE₂ production by 86% (from 38.9 ng/mg to 5.5 ng/mg) and 100% in *C. albicans* and *C. dubliniensis* biofilms, respectively (Figure 5). Sodium azide also significantly inhibited PGE₂ production by 66% (from 38.9 ng/mg to 13.1 ng/mg) for *C. albicans* biofilms and 72% (from 9.9 ng/mg to 2.8 ng/mg) for *C. dubliniensis* biofilms (Figure 5).

A suicide substrate and selective inhibitor of CYP450 epoxygenation reactions, responsible for the production of epoxyeicosatrienoic acids from AA, is 6-(2-propargyloxyphenyl)hexanoic acid (PPOH), a synthetic, acetylenic fatty acid (Wang et al., 1997). Our results indicate that 100 μ M PPOH significantly decreased PGE₂ production by *C. albicans* biofilms by 74% (from 38.9 ng/mg to 10.0 ng/mg), but had no effect on *C. dubliniensis* PGE₂ production (Figure 5). Another suicide substrate used to inhibit CYP450 ω -hydroxylase reactions, responsible for the production of hydroxyeicosatrienoic acids from AA, is 1-aminobenzotriazole (ABT) (Nieves & Moreno, 2006; Su et al., 1998). Our results indicate that ABT at 100 μ M had similar effects to PPOH, causing a 74% (from 38.9 ng/mg to 10.1 ng/mg) and 23% (from 9.9 ng/mg to 7.6 ng/mg) decrease in PGE₂ production by *C. albicans* and *C. dubliniensis* biofilms, respectively (Figure 5). It must however be noted that the specificity of these inhibitors towards certain types of CYP450 reactions was found in mammalian cells and might not be preserved in yeasts. In addition, it is known that these two CYP450 inhibitors also have the ability to reduce PGE₂ production in fibroblast cultures, indicating that it may have an effect on other enzymes such as COX (Nieves & Moreno, 2006). However, the absence of COX enzymes in *C. albicans* and *C. dubliniensis* may limit the action of these inhibitors to CYP450 enzymes and might indicate a role for these enzymes during PGE₂ production by these species, possibly upstream of the multicopper oxidase. None of these inhibitors significantly influenced biomass production or mitochondrial activity of either species (Figures 6 & 7). Although it has been stated in literature that *C. albicans* forms more biofilm than *C. dubliniensis* (Romeo et al., 2011), our results indicate that the *C. albicans* strain used in this study formed significantly less biofilm (measured as dry weight) than the *C. dubliniensis* strain. Similar results were obtained by Henriques and co-workers (2005), suggesting that the observed differences between *C. albicans* and *C. dubliniensis* biofilm formation were due to species and strain variations. Borecká-Melkusová and Bujdaková (2008) indicated that the two genotypes of *C. albicans* (B and C) has a lower ability to form biofilm (measured by cell dry weight and XTT assay) than *C. dubliniensis*.

The known COX and LOX inhibitors, acetylsalicylic acid (ASA) and nordihydroguaiaretic acid (NDGA), were also included in the study. The results indicate that ASA inhibited PGE₂ production by 70-80% in both *C. albicans* and *C. dubliniensis* biofilms (Figure 5) without affecting the biofilm biomass and viability (Figures 6 & 7). Similar results were obtained by Erb-Downward and Noverr (2007) where they found a dose dependant inhibition of PGE₂ production by ASA without influencing the viability of planktonic *C. albicans* cells. However, Alem and Douglas (2005) found that ASA, at 50 µM, inhibited both *C. albicans* biofilm formation and PGE₂ production by more than 20%. Although the mechanism by which ASA, a known COX inhibitor, reduces PGE₂ production by these yeasts is unclear, a possible reason for the reduced levels of PGE₂ in the culture supernatant may be as a result of the inhibition of PGE₂ export by non-steroidal anti-inflammatory drugs rather than inhibition of PGE₂ production (Reid et al., 2003). This will have to be studied in future. Nordihydroguaiaretic acid almost completely inhibited PGE₂ production by both these strains (Figure 5). Erb-Downward and Noverr (2007) found similar results in planktonic *C. albicans* cells and speculated that NDGA may be an alternative substrate for the multicopper oxidase enzyme, since it is a polyphenol with a similar structure to caffeic acid, and later Erb-Downward and co-workers (2008) also found this inhibition of prostaglandin production in *Crypt. neoformans*. In addition, NDGA is an inhibitor of CYP450 monooxygenases (Agarwal et al., 1991; Capdevila et al., 1988) and may therefore inhibit all or most of the enzymes involved in PGE₂ synthesis.

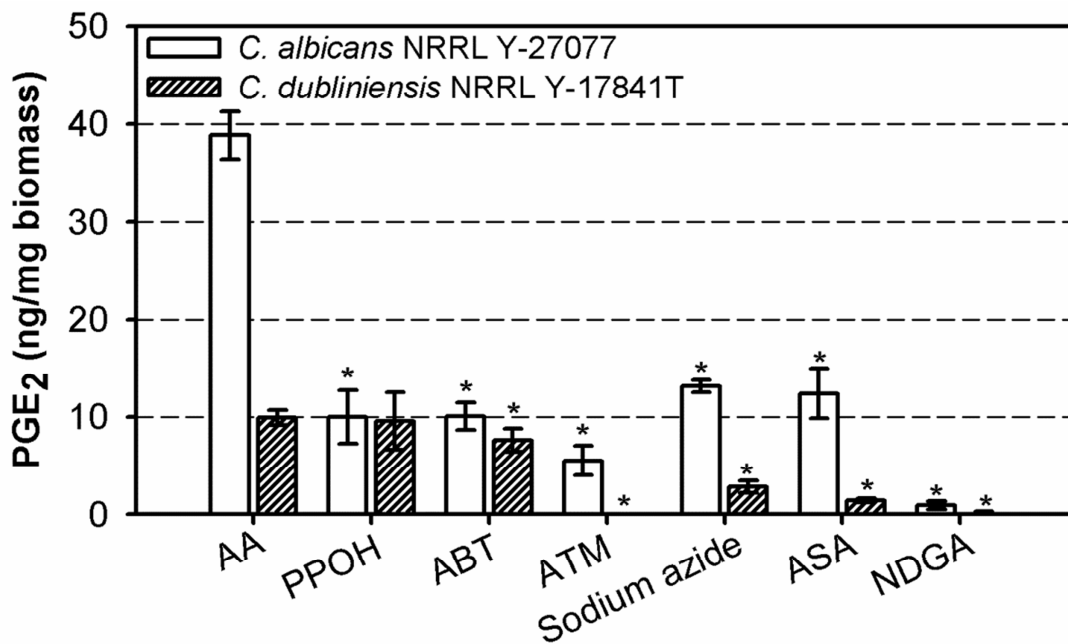


Figure 5. Effect of different inhibitors on extracellular PGE₂ production by *Candida albicans* and *C. dubliniensis* biofilms. All the inhibitors were added at 100 μ M at the same time that the arachidonic acid (AA) was added. Biofilms were incubated at 37°C for 48 h. The concentration PGE₂ in the supernatants was determined as before. ABT, 1-aminobenzotriazole; ASA, acetylsalicylic acid; ATM, ammonium tetrathiomolybdate; NDGA, nordihydroguaiaretic; PPOH, 6-(2-propargyloxyphenyl) hexanoic acid. Values are the mean of duplicate experiments with each sample assayed at two dilutions and each dilution assayed in triplicate and the standard deviation indicated. * Significantly different from control (AA alone) ($P \leq 0.01$).

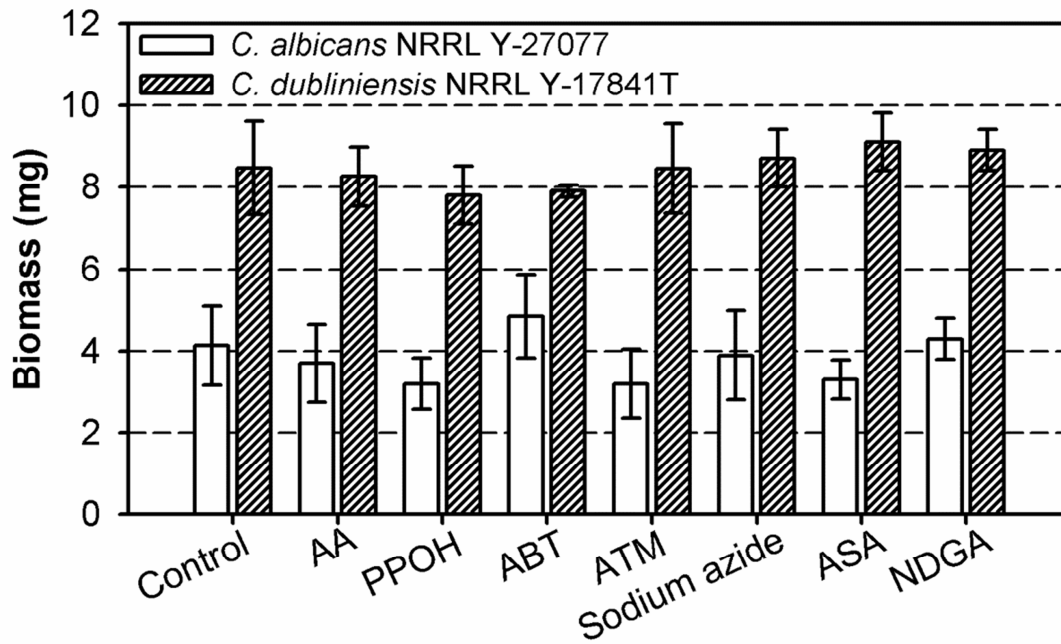


Figure 6. Effect of different inhibitors on biofilm biomass of *Candida albicans* and *C. dubliniensis* biofilms. Inhibitors were added at 100 μ M at the same time that the arachidonic acid (AA) was added. Biofilms were incubated at 37°C for 48 h. The biofilm biomass was evaluated by scraping off the biofilms and filtering it through pre-weighed 0.2 μ m filters. The filters were dried to constant weight for 48 h at 37°C and the biomass determined. ABT, 1-aminobenzotriazole; ASA, acetylsalicylic acid; ATM, ammonium tetrathiomolybdate; NDGA, nordihydroguaiaretic; PPOH, 6-(2-propargyloxyphenyl)hexanoic acid.

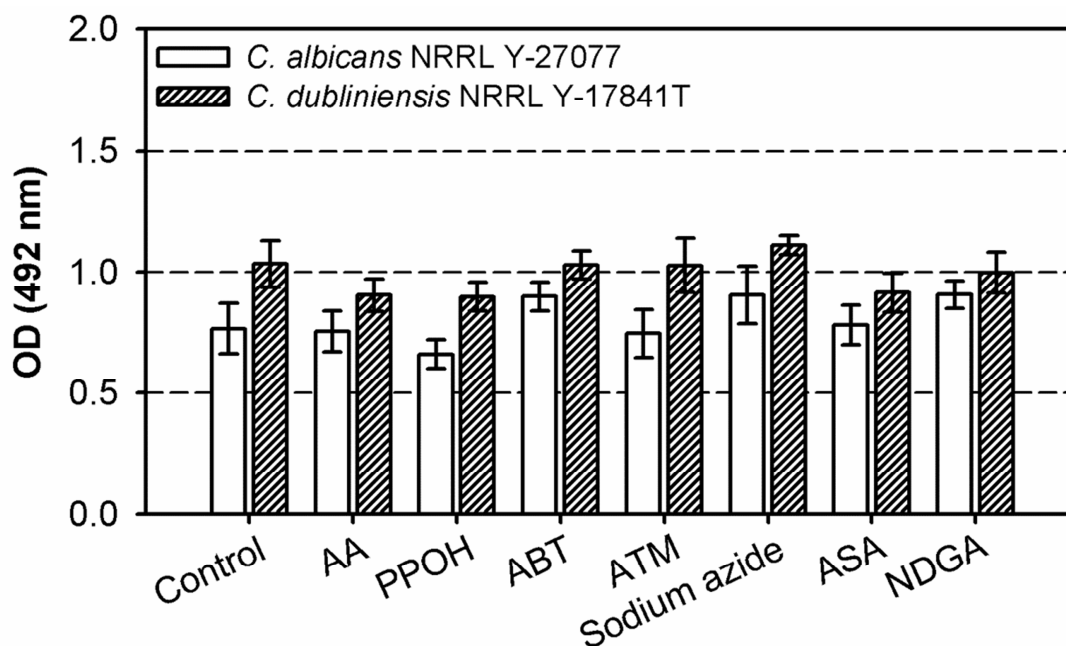


Figure 7. Effect of different inhibitors on mitochondrial metabolism of the biofilms of *Candida albicans* and *C. dubliniensis* biofilms. Inhibitors were added at 100 μ M at the same time that the arachidonic acid (AA) was added and biofilms were incubated at 37°C for 48 h. The mitochondrial metabolism was determined by the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) by mitochondrial enzymes. ABT, 1-aminobenzotriazole; ASA, acetylsalicylic acid; ATM, ammonium tetrathiomolybdate; NDGA, nordihydroguaiaretic; PPOH, 6-(2-propargyloxyphenyl)hexanoic acid.

2.5. Conclusions

This study indicated the production of PGE₂ and PGF_{2 α} by *C. albicans* and *C. dubliniensis* biofilms from exogenous AA. This is also the first report of prostaglandin production by *C. dubliniensis*, as well as PGF_{2 α} by *C. albicans*. The purpose of the production of these prostaglandins by *Candida* species is not fully understood, but it was found that fungal PGE₂ from *C. albicans* and *C. dubliniensis* biofilms were capable of enhancing germ tube formation in both these species. This confirms that it might play an important role as a virulence factor during *Candida* infections. The ability of *C. albicans* to produce more PGE₂ than *C. dubliniensis* might contribute to

the lower level of virulence observed for *C. dubliniensis*. The use of different inhibitors in this study confirmed the involvement of multicopper oxidase enzymes in the production of PGE₂ by *C. albicans* and *C. dubliniensis* biofilms. In addition, inhibitors of CYP (including NDGA) indicate a possible role of these enzymes in prostaglandin production, possibly upstream of the multicopper oxidase. The mechanism by which ASA inhibited PGE₂ production in these biofilms is still unknown, because of the absence of COX enzymes in these *Candida* species.

However, further detailed *in vitro* and *in vivo* studies should be performed with other fungi including other *Candida* species to evaluate the use of CYP450 and multicopper oxidase inhibitors to look for a conserved status, regarding prostaglandin pathways in fungi.

2.6. Acknowledgements

I would like to thank Dr. G. Kemp, from the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, for assistance with the LCMS/MS analysis. I thank the National Research Foundation, South Africa, for funding under the Thuthuka programme (Grant number TTK2007041000014) and the Blue Skies programme (Grant number BS2008092300002).

2.7. References

- Agarwal R, Wang ZY, Bik DP & Mukhtar H. (1991). Nordihydroguaiaretic acid, an inhibitor of lipoxygenase, also inhibits cytochrome P-450 mediated monooxygenase activity in rat epidermal and hepatic microsomes. *Drug Metab Dispos* 19: 620-624.
- Alem MAS & Douglas LJ. (2005). Prostaglandin production during growth of *Candida albicans* biofilms. *J Med Microbiol* 54: 1001-1005.

- Bachmann SP, Vande Walle K, Ramage G, Patterson TF, Wickes BL, Graybill JR & Lopez-Ribot JL. (2002). *In vitro* activity of caspofungin against *Candida albicans* biofilms. *Antimicrob Agents Chemother* 46: 3591-3596.
- Borecká-Melkusová S & Bujdaková H. (2008). Variation of cell surface hydrophobicity and biofilm formation among genotypes of *Candida albicans* and *Candida dubliniensis* under antifungal treatment. *Can J Microbiol* 54: 718-724.
- Brash AR. (2001). Arachidonic acid as a bioactive molecule. *J Clin Invest* 107: 1339-1345.
- Capdevila J, Gil L, Orellana M, Marnett LJ, Mason JI, Yadagiri P & Falck JR. (1988). Inhibitors of cytochrome P-450-dependent arachidonic acid metabolism. *Arch Biochem Biophys* 261: 257-263.
- Deva R, Ciccoli R, Kock JLF & Nigam S. (2001). Involvement of aspirin-sensitive oxylipins in vulvovaginal candidiasis. *FEMS Microbiol Lett* 198: 37-43.
- Deva R, Ciccoli R, Schewe T, Kock JLF & Nigam S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochim Biophys Acta* 1486: 299-311.
- Elmer WK, Stephen DA & William MJ. (1992). Laboratory approach to the diagnosis of fungal infections 14th edn. pp. 387-840. JP Lipincott company, Philadelphia.
- Erb-Downward JR, Noggle RM, Williamson PR & Huffnagle GB. (2008). The role of laccase in prostaglandin production by *Cryptococcus neoformans*. *Mol Microbiol* 68: 1428-1437.
- Erb-Downward JR & Noverr MC. (2007). Characterisation of prostaglandin E₂ production by *Candida albicans*. *Infect Immun* 75: 3498-3505.
- Haas-Stapleton EJ, Lu Y, Hong S, Arita M, Favoreto S, Nigam S, Serhan CN & Agabian N. (2007). *Candida albicans* modulates host defense by biosynthesizing the pro-resolving mediator Resolvin E1. *PLoS ONE* 2(12): e1316. doi:10.1371/journal.pone.0001316.

- Henriques M, Azeredo J & Oliveira R. (2005). *Candida albicans* and *Candida dubliniensis*: comparison of biofilm formation in terms of biomass and activity. *Brit J Biomed Sci* 63: 5-11.
- Imig JD, Falck JR & Inscho EW. (1999). Contribution of cytochrome P450 epoxygenase and hydroxylase pathways to afferent arteriolar autoregulatory responsiveness. *Br J Pharmacol* 127: 1399-1405.
- Johannes C & Majcherczyk A. (2000). Laccase activity tests and laccase inhibitors. *J Biotech* 78: 193-199.
- Nieves D & Moreno JJ. (2006). Hydroxyeicosatetraenoic acids released through the cytochrome P-450 pathway regulate 3T6 fibroblast growth. *J Lipid Res* 47: 2681-2689.
- Noverr MC, Erb-Downward JC & Huffnagle GB. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clin Microbiol Rev* 16: 517-533.
- Noverr MC & Huffnagle GB. (2004). Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect Immun* 72: 6206-6210.
- Noverr MC, Phare SM, Toews GB, Coffey MJ & Huffnagle GB. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* 69: 2957-2963.
- Park J, Lee S, Choi J, Ahn K, Park B, Park J, Kang S & Lee Y-H. (2008). Fungal cytochrome P450 database. *BMC Genomics* 9: 402.
- Ramage G, Vande Walle K, Wickes BL & Lopez-Ribot JL. (2001a). Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 45: 2475-2479.
- Ramage G, Vande Walle K, Wickes BL & Lopez-Ribot JL. (2001b). Biofilm formation by *C. dubliniensis*. *J Clin Microbiol* 39: 3234-3240.
- Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J & Borst P. (2003). The human multidrug resistance protein MRP4 functions as a

prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *P Natl Acad Sci* 100: 9244-9249.

Romeo O, De Leo F & Criseo G. (2011). Adherence ability of *Candida africana*: a comparative study with *Candida albicans* and *Candida dubliniensis*. *Mycoses* 54: doi:10.1111/j.1439-0507.2009.01833.x.

Su P, Maya K & Kroetz DL. (1998). Inhibition of renal arachidonic acid ω -hydroxylase activity with ABT reduces blood pressure in the SHR. *Am J Physiol Regulatory Integrative Comp Physiol* 275: 426-438.

Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE & Coleman DC. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV infected individuals. *Microbiology* 141: 1507-1521.

Wang MH, Brand-Schieber E, Zand BA, Nguyen X, Falck JR, Balu N & Schwartzman ML. (1997). Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: Characterization of selective inhibitors. *J Pharmacol Exp Ther* 284: 966-973.

CHAPTER 3

Sciadonic acid modulates prostaglandin E₂ and cytokine production by epithelial cells during infection with *Candida albicans* and *Candida dubliniensis*

A part of this chapter has been published in Prostaglandins & other Lipid Mediators 97: 66-71, (2012).

3.1. Abstract

Candida albicans is an important opportunistic pathogen in humans causing systemic infections. *Candida* species has the ability to produce pro-inflammatory eicosanoids, such as prostaglandin E₂ (PGE₂), from host derived arachidonic acid (AA) [20:4(n-6)]. This is an important virulence factor. The supplementation of mammalian cells with n-3 fatty acids have been given much attention due to their beneficial health effects, providing immunomodulatory and anti-inflammatory activities. However, uncontrolled production of n-3 fatty acid metabolites could also be detrimental to the host and the high rates of inflammatory and autoimmune diseases are usually due to an imbalance between n-6 and n-3 polyunsaturated fatty acid (PUFA) intake. In addition, mammalian cells cannot produce PGE₂ from non-methylene interrupted fatty acids (NMIFAs), such as sciadonic acid (SA) [20:3(n-6)], providing it with potential anti-inflammatory activities. This led to our hypothesis that the production of PGE₂ and inflammatory cytokines during *Candida* infection can be modulated by incorporation of SA into the host cellular lipids. We demonstrate the incorporation of SA into lipids of epithelial cells. In addition, PGE₂ production was reduced and cytokine profiles influenced in the supplemented infected epithelial cells. Our data suggest that the incorporation of n-6 NMIFAs, SA, might lead to a reduction in both anti- and pro-inflammatory prostaglandins, especially PGE₂, which could benefit the host during a *Candida* infection.

3.2. Introduction

Candida albicans is a dimorphic yeast and the most important opportunistic fungal pathogen, causing infections ranging from mild to life threatening (Ramage et al., 2005). In addition, other non-*albicans Candida* species are also emerging as important opportunistic pathogens (Bahri et al., 2010; Pfaller & Diekema, 2010). One of these pathogens, *C. dubliniensis*, first described in 1995 by Sullivan and co-workers, show a close relationship to *C. albicans* regarding phenotypic characteristics (including germ tube and chlamyospore production) (Ells et al., 2011), but differs from it with respect to epidemiology and certain virulence characteristics (Sullivan et al., 2004). An important virulence factor in *C. albicans* infections is the ability to metabolize the methylene interrupted n-6 long-chain polyunsaturated fatty acid, arachidonic acid (AA) [20:4(n-6)], to pro-inflammatory eicosanoids such as prostaglandin E₂ (PGE₂). It was found by us that *C. dubliniensis* is also capable of metabolizing AA to produce PGE₂, but to a lesser extent compared to *C. albicans* (Chapter 2). Prostaglandin E₂ production is considered a virulence factor, known to promote hyphal formation and infection (Ciccoli et al., 2005; Erb-Downward & Noverr, 2007, Kalo-Klein & Witkin, 1990). Prostaglandin E₂ also plays an important role in host pathogen interactions and the immune response. It has been shown to inhibit T helper cell type 1 (Th1) immune responses, responsible for the evolution of a cell-mediated immunity via inflammation (Chen et al., 2000), chemokine production, mediator release from phagocytes, lymphocyte proliferation (Aronoff et al., 2004; Betz & Fox, 1991; Harizi & Gualde, 2006; van der Pouw Kraan et al., 1995) as well as promoting T helper cell type 2 (Th2) responses [e.g. increased production of interleukin 6 (IL-6)], tissue eosinophilia and immunoglobulin E production (Hinson et al., 1996; Kalinski et al., 1997; Shibata et al., 2005; Snijdwint et al., 1993). As a pro-inflammatory agent, PGE₂ can also induce vasodilatation and enhance oedema in response to inflammatory stimuli (Trang, 1980). Therefore, the importance of this eicosanoid is relevant in this study due to its major role during *Candida* infection as virulence factor and in host pathogen interactions.

During *Candida* infections, innate immunity, provided by the physical barrier of the intact skin and mucosal surfaces, is the first line of defence. However, epithelial

cells also contribute to the immune response by secreting chemokines and cytokines (Hedges et al., 1995; Stadnyk, 1994). Several reports in literature deal with the production of cytokines by epithelial cells upon stimulation with *Candida*. Rouabhia and co-workers (2002) reported that production of interleukin-18 (IL-18) by oral epithelial cells increased upon infection with *C. albicans*, resulting in an increase in gamma interferon (INF- γ) production. In addition, oral epithelial cells also produced increased levels of IL-6 and interleukin-8 (IL-8) when infected with *C. albicans* (Mostefaoui et al., 2004). Steele and Fidel (2002) found that interleukin-1 α (IL-1 α) and tumour necrosis factor alpha (TNF α) were produced by oral and vaginal epithelial cells in response to *C. albicans*. Similarly, Schaller and co-workers (2005) reported that reconstituted human vaginal epithelium produced an increased IL-1 α , interleukin-1 β (IL-1 β), IL-6, IL-8, interleukin-10 (IL-10), granulocyte-macrophage colony-stimulating factor, INF- γ and TNF α response when infected with *C. albicans*. They also observed that the cytokine expression levels correlated with tissue damage. Our understanding of the role of these immune responses to *C. albicans* is still incomplete, but it is generally accepted that pro-inflammatory cytokines may be involved in limiting infection and mounting an appropriate adaptive immune response to the pathogen (Ashman et al., 2011; Dongari-Bagtzoglou & Fidel, 2005). However, uncontrolled activation of pro-inflammatory cytokines may cause increased tissue damage.

It is well known that supplementation of mammalian cells with n-3 fatty acids results in a decrease in pro-inflammatory PGE₂ production and an increase in production of anti-inflammatory 3-series prostaglandins (PGE₃, PGI₃), thromboxane A₃ and prostacyclin (Bagga et al., 2003; Culp et al., 1979; Goodnight et al., 1982) and resolvins (Serhan et al., 2002), resulting in a general anti-inflammatory effect. However, the anti-inflammatory effect of n-6 fatty acids has been limited to γ -linolenic acid (GLA) [18:3(n-6)]. This n-6 fatty acid is not a direct precursor for prostaglandin metabolism, but is converted to dihomo- γ -linolenic acid (DGLA) [20:3(n-6)], also an n-6 fatty acid, which is then converted to anti-inflammatory 1-series prostaglandins and thromboxane A₁ (Kapoor & Huang, 2006).

Interestingly, it was found that sciadonic acid (SA) [20:3(n-6)], a non-methylene interrupted n-6 fatty acid (NMIFA), competes with AA for incorporation

into the phospholipids of mammalian cells, but cannot be directly metabolized to produce prostaglandins (Berger et al., 2002; Berger & Jomard, 2001; Tanaka et al., 2001). This characteristic provides these fatty acids with potential anti-inflammatory properties (Berger & Jomard, 2001; German et al., 1995) and led to the hypothesis that production of PGE₂ and inflammatory cytokines during *Candida* infection can be modulated by incorporation of SA into the host cellular lipids.

3.3. Materials and methods

3.3.1. Host cells and culture conditions

Hep2C epithelial cells, a derivation of cervical epithelial HeLa cells (Moore et al., 1955) considered to be genetically indistinguishable from HeLa cells (Lacroix, 2008), (accession number: 740502, originally obtained from the National Institute for Biological Standards and Control, UK, and maintained by the National Control Laboratories, RSA), were used as an infection model (Deva et al., 2003). Although the cervix is not usually infected by *Candida* spp. due to continuous production of protective mucus by epithelial cells of the endocervical surface (Southern et al., 2008), this protective mechanism is absent in HeLa cells allowing them to be used as an infection model (da Silva et al., 2007; Deva et al., 2003; Moreno-Ruiz et al., 2009; Southern et al., 2008). Cells were cultured at 2×10^5 cells/mL in 75 mm culture flasks (Corning, USA) in minimum essential medium (MEM) containing 4% foetal calf serum (FCS) in the presence of penicillin (100 U/mL) (Invitrogen) and streptomycin (100 µg/mL) (Invitrogen) at 37°C for 24 h until sub confluent layers were formed. These subconfluent cells were further cultured by replacing the media with MEM containing 4% FCS in the presence of penicillin (100 U/mL) and streptomycin (100 µg/mL) and 50 µM SA (Lipidox, Sweden) for 24 h. Sciadonic acid was added to the medium as a bovine serum albumin (BSA) (Sigma Aldrich, USA) complex at a molar ratio of 3:1 (Tanaka et al., 2001). As control, an equivalent amount of BSA was added to the subconfluent cells.

3.3.2. Epithelial cell lipid analysis

After 24 h incubation at 37°C, the Hep2C cells were rinsed three times with phosphate buffered saline (PBS) (w/o calcium and magnesium) before they were scraped from the plates, the wet biomass determined and total lipids extracted by the use of chloroform:methanol (2:1) (Folch et al., 1957). The total lipids were separated into different fractions by the use of solid phase 0.50 g Si extraction columns (Separations, RSA) according to the method of Bossio and Scow (1998). Briefly, columns were conditioned with 2 mL chloroform followed by transfer of the lipid samples, resuspended in 300 µL chloroform, to the columns. The neutral lipids were eluted with 5 mL chloroform, followed by the glycolipids with 10 mL acetone and finally the phospholipids with 5 mL methanol. The eluates were collected and dried under N₂ at 32°C. Fatty acid methyl esters (FAMES) of the lipid fractions were prepared using methanol-BF₃ (Diaz et al., 2005; Hur et al., 2004; Slover & Lanza, 1979) and quantified using a Varian GX 3400 gas chromatograph, with a fused silica capillary column (Chrompack CPSIL 88, 100 m length, 0.25 mm ID, 0.2 µm film thickness). The column temperature was 40–230°C (hold 2 minutes; 4°C/minute; hold 10 minutes). Fatty acid methyl esters in hexane (1 µL) were injected into the column using a Varian 8200 CX Autosampler with a split ratio of 100:1. The injection port and detector temperatures were maintained at 250°C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the makeup gas. Varian Star Chromatography Software recorded the chromatograms. Fatty acid methyl ester samples were identified by comparing the relative retention times of FAME peaks from samples with those of standards. Fatty acids were expressed as the relative percentage of each individual fatty acid to the total of all fatty acids present in the sample. This experiment was performed in duplicate.

3.3.3. Yeast strains used

The following clinical strains were used in this study: *Candida albicans* NRRL Y-27077 (isolated from a skin lesion, Germany) and *C. dubliniensis* NRRL Y-17841T (isolated from an HIV patient, Dublin, Ireland). Both strains were obtained from the Agricultural Research Service Culture Collection of the United States Department of

Agriculture. All strains were maintained on Yeast Malt Extract (YM) agar (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 16 g/L agar) at room temperature.

3.3.4. Infection of Hep2C cells with *Candida* species

The media were removed from cells supplemented with SA and replaced with MEM containing 0% FCS in the presence of penicillin (100 U/ml) and streptomycin (100 µg/mL). These cells were then infected with 1×10^6 cells/mL *C. albicans* or *C. dubliniensis*, grown in synthetic media (6.7 g/L Yeast Nitrogen Base) containing 500 mM glucose for 24 h at 37°C aerobically, and incubated at 37°C up to 20 h. Samples were taken after 1, 10 and 20 h, and the supernatants centrifuged (10 min at 4412 x g) (Heraeus, Megafuge 1R) and stored at -80°C until further analysis.

3.3.5. Scanning electron microscopy (SEM)

Hep2C cells were grown in flat bottomed cell culture tubes (Nunc, Denmark) containing sterile glass coverslips and treated as above. After incubation for 10 h at 37°C, the coverslips were carefully rinsed with sterile PBS (OXOID, UK). The coverslips containing Hep2C and *Candida* cells were fixed with 3% (v/v) gluteraldehyde (primary fixative) (Merck, Darmstadt, Germany) in phosphate buffer (pH 7.0) overnight at room temperature. After washing the coverslips carefully with phosphate buffer, the cells were fixed with 0.5% (v/v) osmiumtetroxide (secondary fixative) (Merck, Darmstadt, Germany) in phosphate buffer (pH 7.0) for 1 h at room temperature. The dehydration steps were performed with different ethanol concentrations (50%, 70%, 95%) for 15 min each and then finally twice with absolute ethanol for 60 min. After the cells had undergone critical point drying, the glass coverslips were mounted on metal stubs and coated with gold. The cells were then examined with a Shimadzu SSX-550 Superscan scanning electron microscope (SEM).

3.3.6. Nano Scanning Auger Microscopy (NanoSAM)

The samples prepared for SEM, were also examined using a PHI 700 Nanoprobe (Japan) equipped with SAM and SEM facilities (Swart et al., 2010). Two targets were selected to analyze and denoted as target 1, the nodule on epithelial cell, and target 2, the epithelial cell. The field emission electron gun used for the SEM and SAM analyses was set at 2.34 A filament current, 4 kV extractor voltage and 240.6 μ A extractor current. With these settings a 25 kV, 10 nA electron beam was obtained for the Auger analyses and SEM imaging. The electron beam diameter was 12 nm. The upper pressure of the electron gun unit was 1.24×10^{-9} Torr. The pressure in the main chamber was 6.07×10^{-10} Torr. Aperture A was used for all the measurements. The Field of View (FOV) for SEM was 2 μ m and the number of frames used was 1 on a slow scan speed. The Auger point analyses were obtained by using 10 cycles per survey, 1 eV/step and 50 ms per step. The Nanoprobe was also equipped with an Ar⁺ ion sputtering gun set at 2 kV beam voltage, 2 μ A ion beam current and a 2 x 2 mm raster area, giving a sputter rate of ~ 8.5 nm/min. The ion emission current was set at 15 mA. An alternating sputter mode with sputter intervals of 1 min and sputter time of 2 min was used without any rotation.

3.3.7. Determination of prostaglandin E₂ production by *Candida* biofilms

Candida albicans and *C. dubliniensis* biofilms were grown in filter sterilized RPMI-1640 medium (Sigma Aldrich, USA) containing a final concentration of 500 μ M AA (Sigma Aldrich, USA) or 500 μ M SA (in 0.38% v/v ethanol) at a cell concentration of 1×10^6 cells/mL. Appropriate controls were included. These suspensions were dispensed into 90 mm polystyrene Petri dishes (Merck, RSA) and incubated at 37°C for up to 48 h, to allow biofilm formation. The supernatants from the biofilms were harvested, centrifuged (10 min at 4412 x g) and filtered through 0.2 μ m cellulose acetate syringe filters (GEMA Medical SL, Spain). The supernatants for PGE₂ determination were then purified by the use of a PGE₂ affinity column according to the manufacturer's instructions (Cayman Chemicals, USA). The concentration PGE₂ was then determined by monoclonal PGE₂ enzyme-linked immunosorbent assay (ELISA) (Cayman Chemicals, USA) according to the manufacturer's instructions.

The ELISA kit is highly sensitive and detects as little as 15 pg/mL of PGE₂. Controls included biofilms grown without AA or SA and RPMI-1640 medium with AA or SA alone. Background levels of PGE₂ detected in medium with AA or SA alone were subtracted from the experimental samples. The experiment was done in duplicate, with each sample assayed at two dilutions and each dilution assayed in triplicate.

3.3.8. Determination of biomass and cell viability of *Candida* biofilms

The effect of growth in the presence of 500 μM AA and SA on biofilm biomass was evaluated by scraping of the biofilms from above and determining the wet biomass. The effect of 500 μM AA and SA on cell viability of the biofilms was studied using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma Chemicals, USA). This experiment was performed in triplicate.

3.3.9. Determination of prostaglandin E₂ production by epithelial cells in response to *Candida*

The stored supernatants were used to determine PGE₂ concentration released by epithelial cells, by the use of a monoclonal PGE₂ ELISA as described above. Appropriate controls were included. Background levels of PGE₂ detected in medium with SA alone was subtracted from the experimental samples. The experiment was done in duplicate, with each sample assayed at two dilutions and each dilution assayed in triplicate.

3.3.10. Determination of cytokines produced by epithelial cells in response to *Candida*

The stored supernatants were assayed for INF-γ (detection limit: 10 pg/mL), IL-1α (detection limit: 2 pg/mL), TNF-α (detection limit: 2 pg/mL), IL-1β (detection limit: 2 pg/mL), IL-4 (detection limit: 2 pg/mL), IL-6 (detection limit: 2 pg/mL), IL-10 (detection limit: 2 pg/mL) and IL-13 (detection limit: 1 pg/mL) as well as chemotactic

cytokines [IL-8 (detection limit: 0 pg/mL) and monocyte chemoattractant protein 1 (detection limit: 2 pg/mL) (MCP-1)], by using cytokine antibody arrays according to the manufactures instructions (Quantibody[®] Human Inflammation Array 1, Raybiotech, Inc. USA). These are quantitative arrays based on multiplexed sandwich ELISA technology to accurately determine the concentration of cytokines (Piganelli & Mathews, 2007). Briefly, the glass chips were covered with 100 μ L sample diluent and incubated for 30 min, followed by washing. Each well, arrayed with cytokine antibodies, were overlaid with 100 μ L of standard cytokine or sample supernatant containing cytokines. This was done in duplicate, with each cytokine arrayed in quadruplicate per slide. After overnight incubation at 4°C and extensive washing, the detector antibody was added and incubated overnight at 4°C, followed by washing again. This is followed by the addition of the streptavidin-labeled Cy3 equivalent dye and incubated overnight at 4°C. After extensive was hing, the signals were scanned with a GenePix 4000B laser scanner (Axon Instruments Inc., Foster City, CA, USA). Cytokines were quantified with GenePix Pro 6.0 software program (Axon Instruments / Molecular Devices Corp., USA) against a standard curve set for each cytokine, with a five-point serial dilution of cytokine standards from the same array.

3.3.11. Statistical analysis

All experiments were performed at least in duplicate unless stated otherwise. In cases where three or more values were obtained, the *t*-test was performed to determine the significance of the data sets.

3.3.12. Ethics approval

No ethics approval was required for this study, since the Hep2C cell line was not a *de novo* cell line and both the yeast strains used in this study are commercial strains obtained from an open access yeast culture collection.

3.4. Results and discussion

3.4.1. Sciadonic acid is incorporated into epithelial cell lipids

As expected, a considerable amount of SA is incorporated into the neutral [38.9% (\pm 3.1)] (Figure 1a) and phospholipids [20.8% (\pm 2.1)] (Figure 1b). It must, however, be noted that any free SA merely adhering to the cell surfaces will elute with the neutral lipids. Interestingly, the percentage AA decreased from 3.0% (\pm 1.3) to 1.5% (\pm 0.3) in the phospholipids of the SA supplemented epithelial cells. This might indicate the substitution of AA in the phospholipids of these cells by SA (Tanaka et al., 1999, 2001). The incorporation of SA also influenced other fatty acids in both neutral and phospholipids. Interestingly, there is an increase in linoleic acid [18:2(n-6)] in the neutral lipids [from 2.2% (\pm 0.1) to 8.7% (\pm 0.6)], of SA supplemented epithelial cells. Palmitoleic acid [16:1(n-7)] decreased from 5.4% (\pm 0.9) to 2.4% (\pm 0.5) in the neutral lipids and from 8.0% (\pm 0.9) to 3.7% (\pm 0.2) in the phospholipids. Stearic acid (18:0) decreased [from 7.1% (\pm 2.0) to 2.3% (\pm 0.3)] only in the neutral lipids, whereas oleic acid [18:1(n-9)] decreased in the neutral lipids [from 17.5% (\pm 1.6) to 9.4% (\pm 2.3)] and in the phospholipids [from 20.8% (\pm 1.4) to 10.6% (\pm 1.5)]. In addition, cis-vaccenic acid [18:1(n-11)], decreased in the neutral lipids [from 10.7% (\pm 2.5) to 3.7% (\pm 0.4)] as well as in the phospholipids [from 13.5% (\pm 0.6) to 7.5% (\pm 0.2)]. The other fatty acids detected, including the n-3 fatty acids, eicosapentaenoic acid (EPA) [20:5(n-3), and docosahexaenoic acid (DHA) [22:6(n-3), were not significantly affected by the incorporation of SA.

This indicates that we were able to modify the lipid composition of Hep2C epithelial cells by incorporating SA into both the neutral and phospholipids. Interestingly, the decrease in AA concentration, due to the incorporation of SA, was only found in the phospholipids but not in the neutral lipids. Previously, Tanaka and co-workers (1999, 2001) indicated that SA is capable of substituting AA in the phospholipids, specifically in the phosphatidylinositol fraction. They also found that AA cannot be directly synthesized from SA, but that it rather mimics AA in the biosynthesis of phospholipids. Huang and co-workers (2011) found an increase in SA and AA as well as other n-6 polyunsaturated fatty acids (PUFAs) in cellular phospholipids when macrophages were supplemented with eicosadienoic acid [20:2(n-6)], an unusual n-6 PUFA which is a precursor for SA and/or AA synthesis.

Although Endo and co-workers (2009) found that SA metabolism in rat liver inhibits AA production from 18:2(n-6), other authors indicated that SA may be metabolized to palmitolinoleic acid [16:2(n-6)] and then to 18:2(n-6) and AA through a sequence of reactions, including chain shortening and elongation (Tanaka et al., 2007). This process is referred to as PUFA remodelling which is necessary for the conversion of unsuitable fatty acids into essential fatty acids. Although we did not observe an increase in 16:2(n-6), an increase in 18:2(n-6) was observed in the neutral lipids upon SA incorporation. This may be explained by the rapid conversion of 16:2(n-6) to 18:2(n-6).

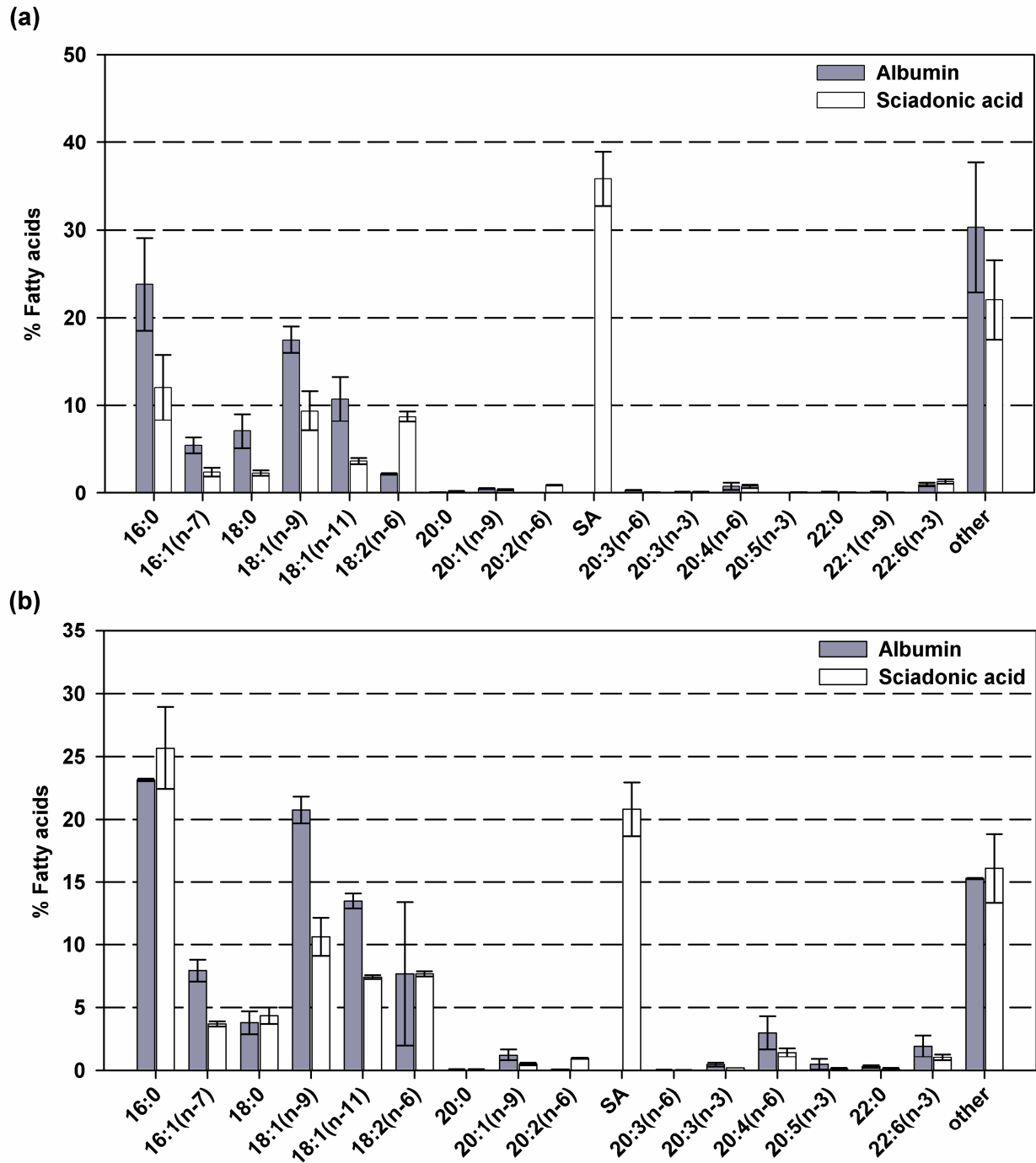


Figure 1. The percentage fatty acids in Hep2C cells were determined by gas chromatography after incubation for 24 h at 37°C in the absence and presence of 50 μ M sciadonic acid (SA). (a) The percentage fatty acids in the neutral lipids. (b) The percentage fatty acids in the phospholipids. Values are the mean of duplicate experiments and the range is indicated by error bars.

3.4.2. Microscopical analyses of epithelial cells during infection

The initial step in both commensal colonization and infections caused by *Candida* involves adhesion to the epithelial cells (Yang, 2003). An important factor for attachment to epithelial cells is the conversion of yeast cells to hyphal structures, where the hyphal form is generally associated with attachment leading to infections (Villar et al., 2004). In addition, Filler & Sheppard (2006) and Tsarfaty and co-workers (2000) noted that both *C. albicans* yeast and hyphal forms were responsible for initiation and development of infections. Similarly, Lim and co-workers (2011) also found that high densities of *C. albicans* yeast cells, contributed to the pathogenesis in human umbilical vein endothelial cells.

Scanning electron microscopy was used to visualize the morphology of *Candida* species and Hep2C epithelial cells during infection. Although *C. albicans* did not produce hyphal structures as *C. dubliniensis*, which formed pseudohyphal structures, both morphological forms were able to grow into and between the cells (Figure 2). It can also be seen that the epithelial cells produced microvilli-like structures (Dalle et al., 2010), in response to *Candida* infections. According to literature, epithelial cells produce structures in response to *C. albicans* that enfold and attach the yeast to the epithelial cells, which could also lead to invasion into the epithelial cell (Phan et al., 2007; Zakikhany et al., 2007). Interestingly with SA supplemented epithelial cells in response to *C. dubliniensis* (Figure 2d), we saw not only microvilli-like structures but also nodules on the surface of the epithelial cells. These nodules are not spherical but have a non-homogeneous structure.

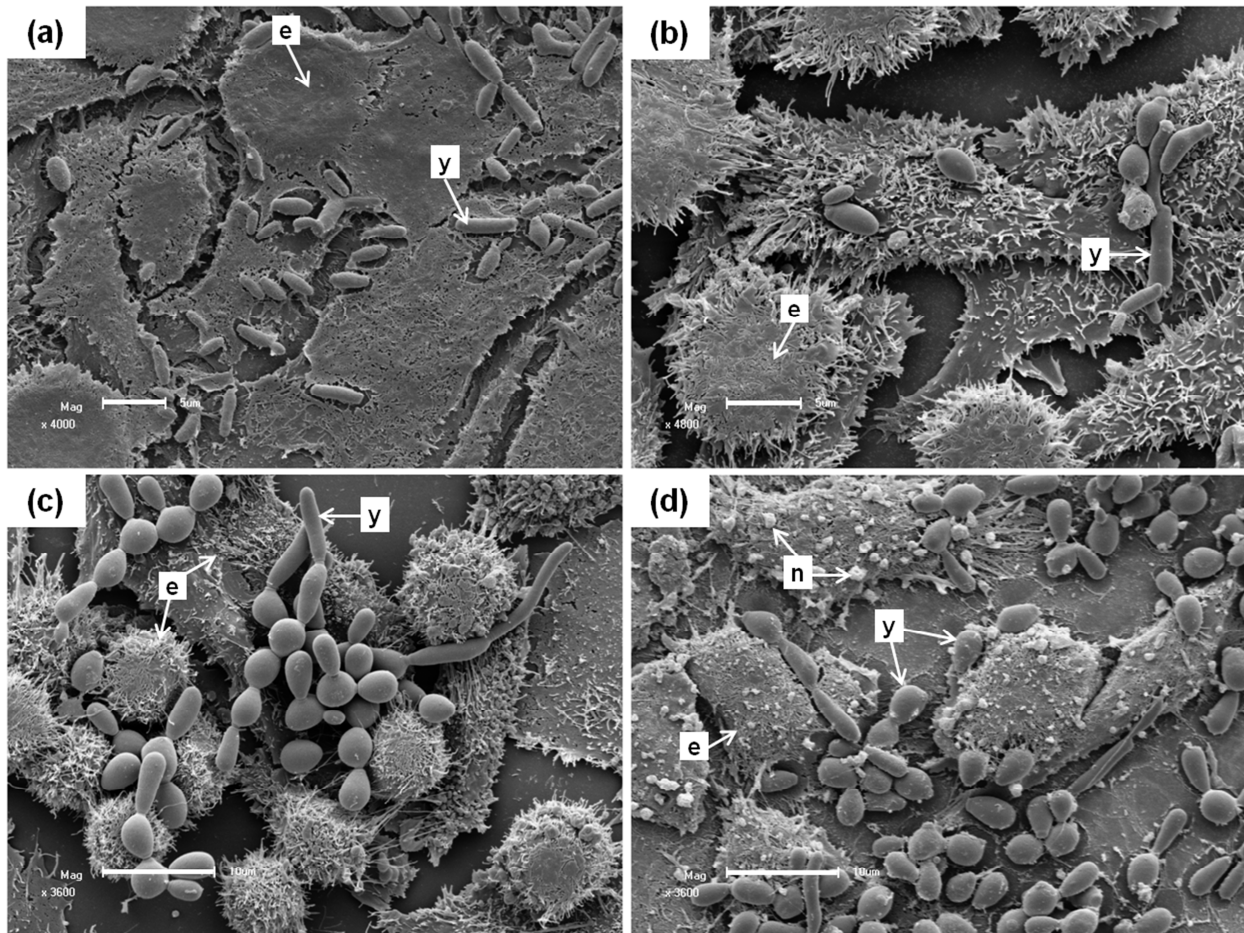


Figure 2. Scanning electron micrographs showing the attachment of *Candida* species to supplemented and unsupplemented Hep2C cells after 10 h of infection at 37°C. (a) *C. albicans* NRRL Y-27077 attached to unsupplemented Hep2C cells (scale bar = 5 μm). (b) *C. albicans* NRRL Y-27077 attached to Hep2C cells supplemented with 50 μM sciadonic acid (scale bar = 5 μm). (c) *C. dubliniensis* NRRL Y-17841T attached to unsupplemented Hep2C cells (scale bar = 10 μm). (d) *C. dubliniensis* NRRL Y-17841T attached to Hep2C cells supplemented with 50 μM sciadonic acid (scale bar = 10 μm). e: epithelial cell; n: nodule; y: yeast

The SEM samples of the SA supplemented epithelial cells, infected with *C. dubliniensis*, were further analyzed by Nano Scanning Auger Microscopy (NanoSAM) to try and characterize the nodules present on the surfaces of the epithelial cells. Figure 3 indicates that these nodules are approximately 0.5 μm in diameter, suggesting that it is much smaller than the necrotic or apoptotic blebs described by Barros and co-workers (2003) for HeLa cells. Therefore, these nodules are probably not epithelial blebs. To confirm this, the elemental analyses shown as percentage atomic concentration (Figure 4), indicates that the two targets denoted as target 1 (the nodule) and target 2 (the epithelial cell) have different atomic ratios. We therefore hypothesized that the nodule is most probably the fatty acid BSA complex that is attached to the outside of the epithelial cells, before being taken up. The proposed structure is illustrated in Figure 5 where BSA forms a hydrophilic micelle, similar to the casein micelle of bovine milk (Phadungath, 2005), consisting out of small BSA sub-micelles (also to be seen in Figures 3e, f, during sequential etching). Each sub-micelle (Figure 5b) consists out of albumin with the SA bound to it (Figure 5c). It is known that hydrophobic insoluble fatty acids can bind to the albumin through hydrophobic interactions (Choi et al., 2002; Spector, 1975). More specifically, the protein chain surrounds the carbon-rich tails of the fatty acids, thus protecting them from the surrounding aqueous environment. It is known that human serum albumin, which has a 75% sequence identity to BSA, can bind up to seven AA molecules (Curry et al., 1999; Petitpas et al., 2001). The percentage atomic concentration after 4 and 8 min of sequential etching through the nodule (Figure 4a), indicates a carbon to oxygen ratio of approximately 10:1, which is representative of SA, indicating the presence of SA inside this nodule. The increase in the gold concentration at the beginning and after 5 min of etching (Figure 4a), might suggest that the nodule surface is not smooth or that it consists out of smaller subunits packed together with the gold coated into the crevices between these subunits.

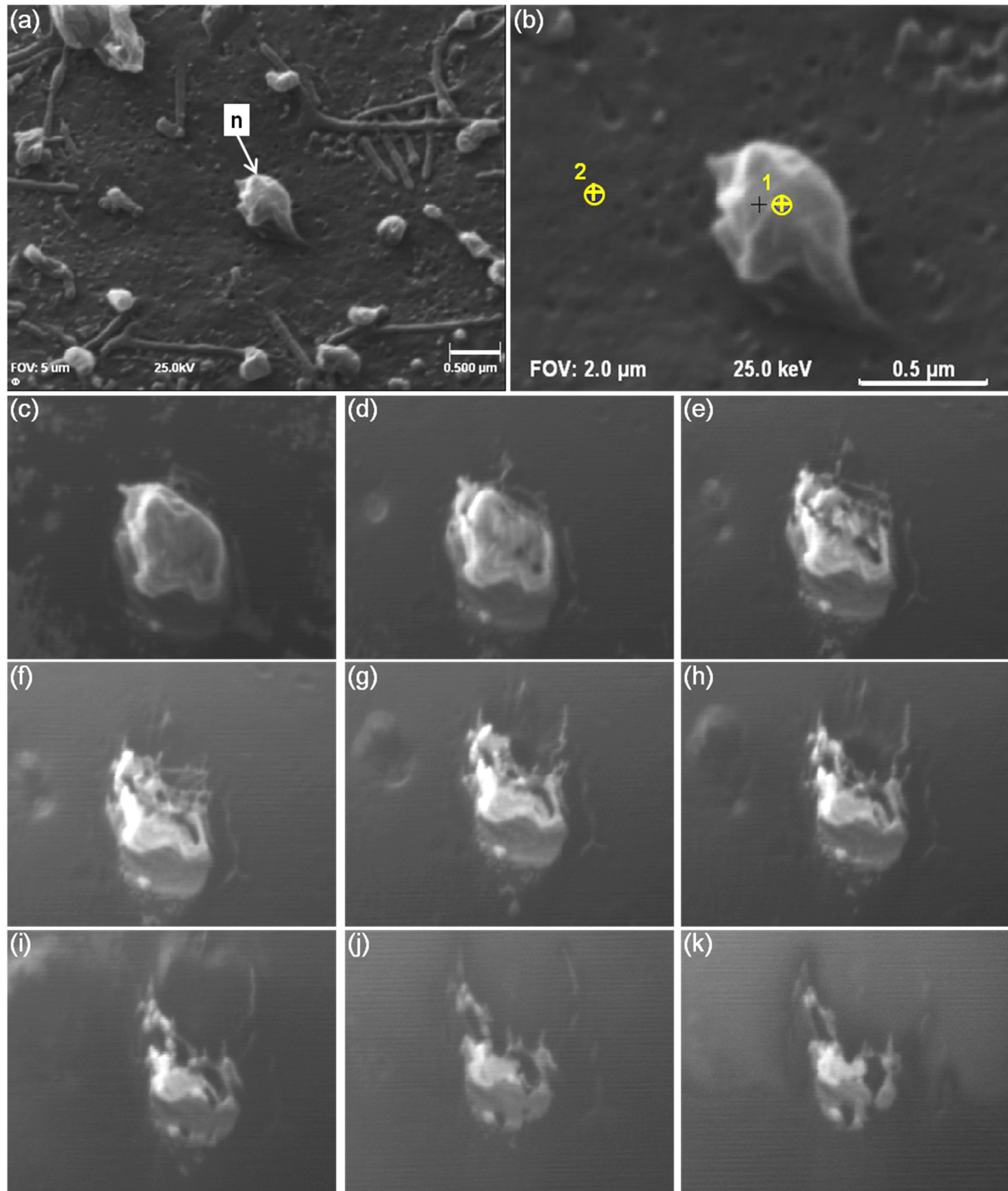


Figure 3. (a) Scanning electron micrographs of Hep2C epithelial cells indicating the nodules (n) during sciadonic acid treatment and *C. dubliniensis* infection. (b) Magnification of a nodule as well as the targets for elemental analyses, indicated by the crossed circles, target 1 is the nodule and target 2 the epithelial cell. (c – k) Scanning electron micrographs at different stages of sequential targeted etching during 1 min intervals into the nodule (target 1). (scale bar = 0.5 μm)

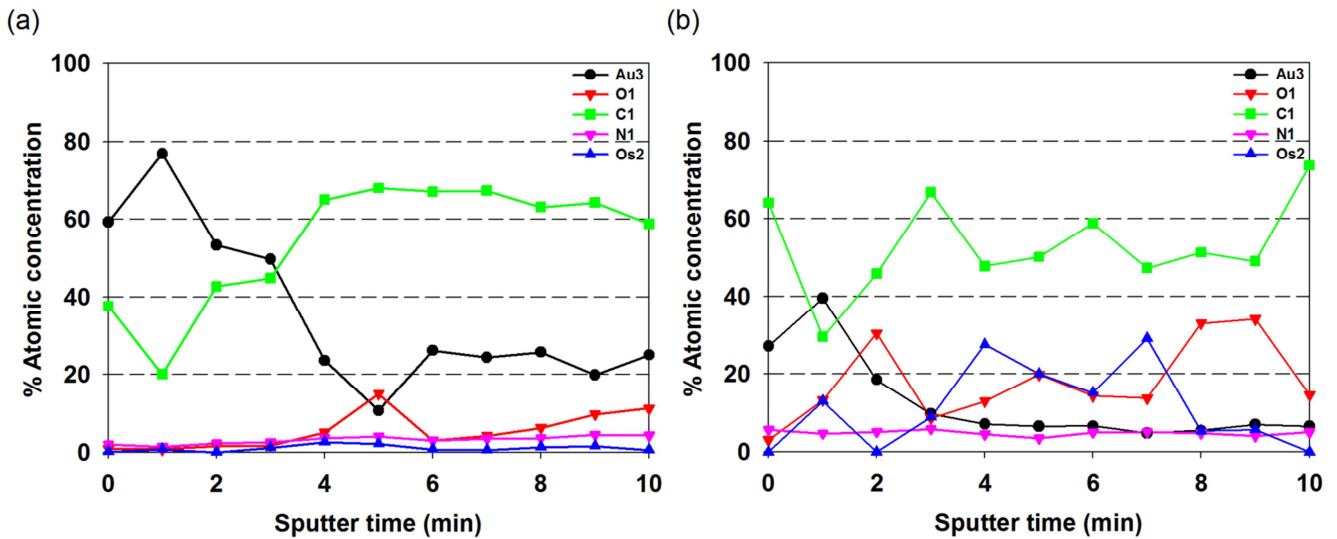


Figure 4. Elemental analyses through the nodule and the Hep2C epithelial cells during sequential etching. (a) Graph showing atomic concentration over sputter time of the nodule (target 1). (b) Graph showing atomic concentration over sputter time of the epithelial cells (target 2).

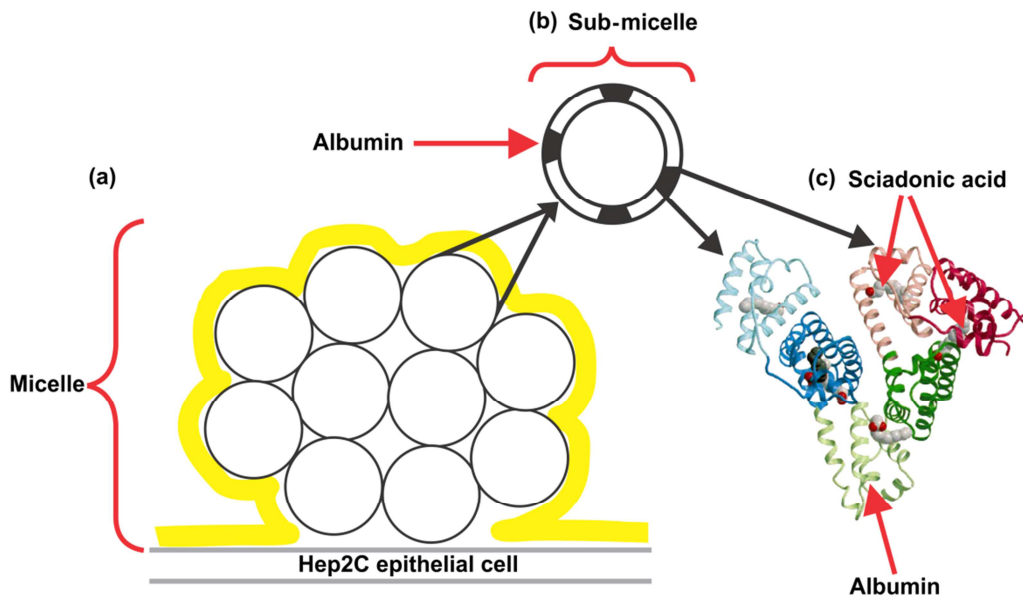


Figure 5. Schematic diagram of the proposed structure of the observed nodule on Hep2C epithelial cells after sciadonic acid treatment. (a) The nodule attached to the Hep2C cells covered with gold represents the micelle. (b) The micelle is composed of sub-micelles consisting out of albumin molecules. (c) The albumin molecule with the bound sciadonic acid.

3.4.3. Sciadonic acid modifies prostaglandin E_2 production by *C. albicans*, *C. dubliniensis* and infected epithelial cells

Similar to mammalian cells (Berger et al., 2002), *C. albicans* and *C. dubliniensis* are incapable of metabolizing SA directly to PGE_2 (Figure 6). This is in contrast to the high level of PGE_2 produced from exogenous AA. The AA and SA did not have a significant effect on biofilm biomass (Figure 7) and viability (Figure 8) of both these strains. Figure 9 indicates that *C. albicans* and *C. dubliniensis* are both capable of stimulating similar levels of PGE_2 production by Hep2C cells from as early as 1 h of infection, i.e. 26.4 (\pm 5.6) pg/mL and 23.5 (\pm 4.4) pg/mL, respectively. During *C. albicans* infection, both host and yeast cells contribute to prostaglandin, especially PGE_2 , production (Ciccoli et al., 2005; Erb-Downward & Noverr, 2007). Deva and co-workers (2001, 2003) indicated that *C. albicans* stimulates PGE_2 production by HeLa cells by inducing cyclooxygenase 2 (COX-2) in these cells. This induction was found to be in response to β -glucan, a cell wall component of *C. albicans* and a major fungal pathogen associated molecular pattern (Castro et al., 1994; Suram et al., 2010). The stimulation of PGE_2 production by *C. albicans* was not only found in epithelial cells but also in endothelial and peripheral blood mononuclear cells (Filler et al., 1994; Witkin et al., 1991).

Interestingly, in the presence of SA, there was a significant decrease in PGE_2 production in response to both *Candida* species after 1 h and 10 h of infection. During *C. albicans* infection of SA supplemented cells, a decrease of 68% [from 26.4 (\pm 5.6) pg/mL to 8.4 (\pm 1.4) pg/mL] was observed after 1 h and a decrease of 50% [from 20.2 (\pm 3.1) pg/mL to 10.2 (\pm 1.6) pg/mL] after 10 h of infection. During *C. dubliniensis* infection of SA supplemented cells, a decrease of 29% [from 23.5 (\pm 4.4) pg/mL to 16.7 (\pm 1.7) pg/mL] was observed after 1 h of infection and a decrease of 11% [from 19.9 (\pm 1.3) pg/mL to 17.7 (\pm 2.7) pg/mL] after 10 h of infection. This decrease in extracellular PGE_2 concentration in response to *Candida* infection of epithelial cells supplemented with SA might partly be explained by the inability of these *Candida* species to produce PGE_2 from exogenous SA, thus decreasing yeast derived PGE_2 from the infection model. In addition, the ability of SA to replace AA in the host phospholipids (Huang et al., 2011; Tanaka et al., 2001), results in less AA available for host and yeast derived PGE_2 production. This, together with the inability

of mammalian cells to directly metabolize SA to prostaglandins (Berger et al., 2002) and possible substrate competition between SA and AA for COX-2 (Chuang et al., 2009), may contribute to the observed decrease. The observed decrease in PGE₂ was also found in macrophages supplemented with SA when stimulated with lipopolysaccharide (LPS) (Huang et al., 2011).

Interestingly, after 20 h infection of SA supplemented cells, there was a significant increase in PGE₂ concentration compared to the unsupplemented infected epithelial cells, from 13.7 (\pm 1.9) pg/mL to 17.9 (\pm 3.9) pg/mL and from 14.9 (\pm 0.9) pg/mL to 22.4 (\pm 4.6) pg/mL in response to both yeasts. The SA incorporated into the phospholipids of the epithelial cells can be metabolized to produce 18:2(n-6) (Tanaka et al., 2007). This could then lead to the production of AA, from 18:2(n-6), followed by the production of PGE₂ (Isseroff et al., 1987). This might explain the observed delayed increase in extracellular PGE₂ concentration in the supplemented epithelial cells over the infection period in response to both yeasts. The decrease in PGE₂ concentration from 1-20 h in unsupplemented host cells may be explained by the depletion of AA from the host phospholipids, leading to less AA being available for conversion to PGE₂.

The mechanism involved in yeast PGE₂ production by *Candida* is poorly understood. However, during an infection it is proposed that *C. albicans* secretes phospholipases into the environment (Smith, 1989) or stimulates the activation of host cytosolic phospholipases (Suram et al., 2010), resulting in the release of AA from host phospholipids. This AA can serve as substrate for PGE₂ synthesis (Noverr et al., 2001) as well as formation of 3(*R*)-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (Deva et al., 2001). Additionally, Ciccoli and co-workers (2005) indicated that a 3-hydroxyeicosatetraenoic acid can be used as a substrate for the production of the inflammatory 3-hydroxy-prostaglandin E₂ (3-OH PGE₂) by mammalian COX-2 in a HeLa infection model. Although studies have shown that acetyl salicylic acid, indomethacin as well as cytochrome P450 inhibitors reduce the production of PGE₂ in *C. albicans* and *C. dubliniensis* (Chapter 2), the enzymes responsible for this activity remain to be identified (Ciccoli et al., 2005; Deva et al., 2001; Noverr et al., 2001, 2002). Similarly, the exact role of PGE₂ during yeast infection is not fully understood. It is known that PGE₂ could be a virulence factor as it is able to enhance

germ tube formation of both *C. albicans* and *C. dubliniensis* and plays a role in the host immune response (Ciccoli et al., 2005; Erb-Downward & Noverr, 2007; Noverr et al., 2002). In addition, women with recurrent vulvovaginal candidosis have a localized immunosuppression due to increased levels of PGE₂ leading to proliferation of *Candida* (Weissenbacher et al., 2009). Thus inhibition of PGE₂ production may be important in controlling colonization and infection.

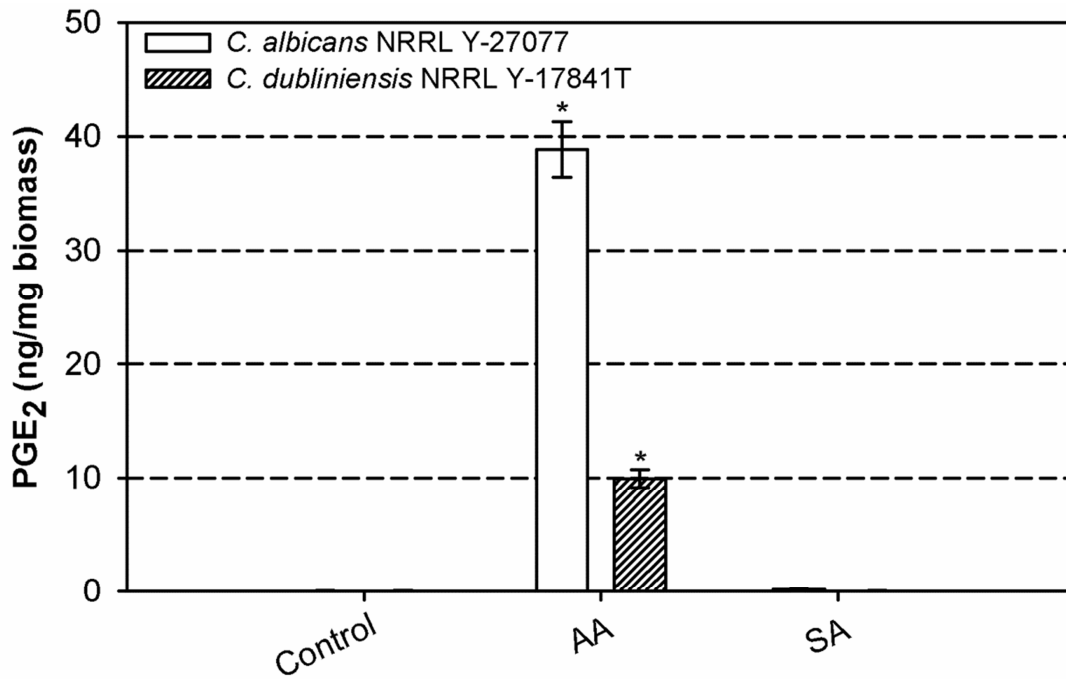


Figure 6. Prostaglandin E₂ production by *Candida albicans* and *C. dubliniensis* biofilms. Biofilms were grown for 48 h at 37°C in the absence and presence of 500 μM arachidonic acid (AA) or 500 μM sciadonic acid (SA). PGE₂ production was determined by the use of a monoclonal PGE₂ EIA kit (Cayman Chemicals). Values are the mean of duplicate experiments with each sample assayed at two dilutions and each dilution assayed in triplicate and the standard deviation indicated. * Significantly different from control ($P \leq 0.01$).

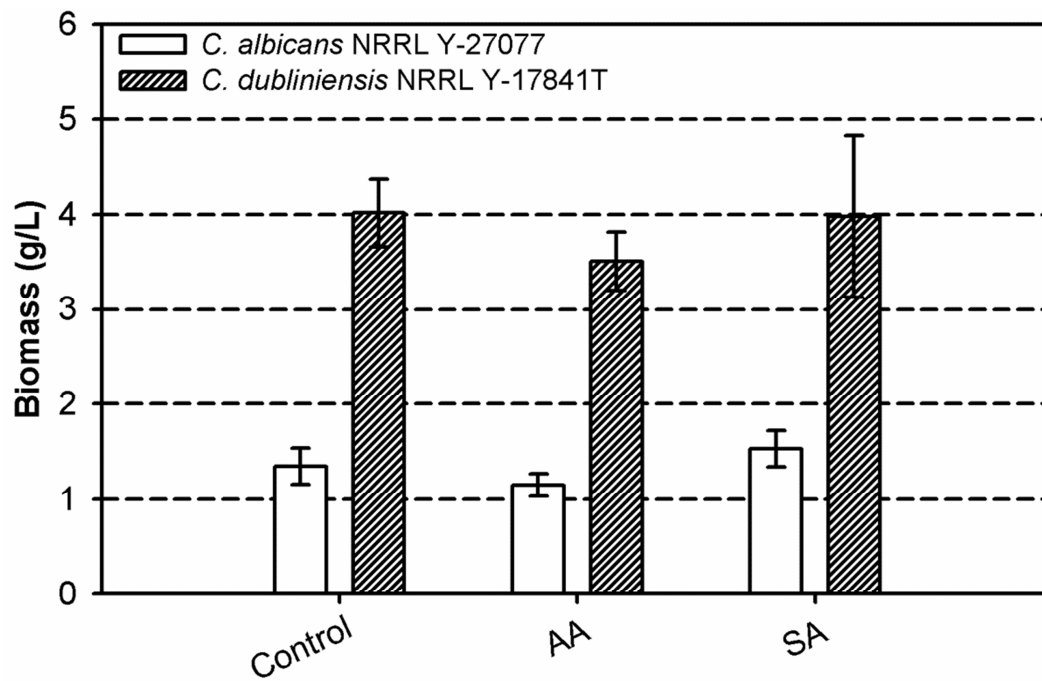


Figure 7. Effect of arachidonic (AA) and sciadonic acid (SA) on biofilm biomass of *Candida albicans* and *C. dubliniensis* biofilms. The fatty acids were used at 500 μ M. The biofilms were obtained from the plates used for PGE₂ determination and the wet biomass of the biofilms determined.

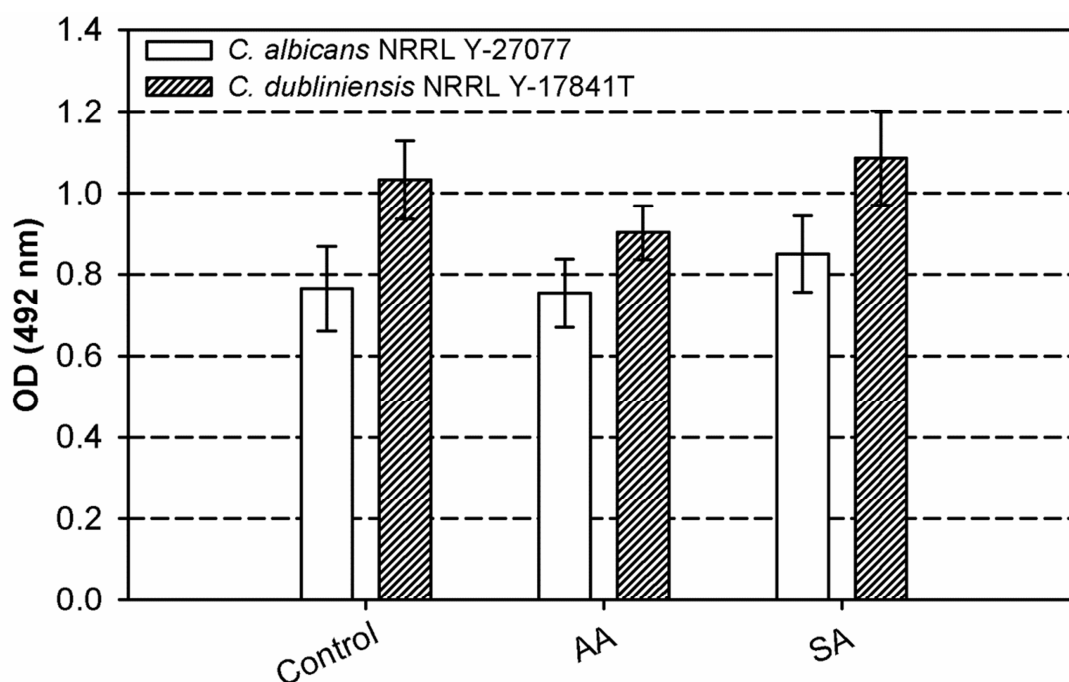


Figure 8. Effect of arachidonic (AA) and sciadonic acid (SA) on mitochondrial metabolism of the biofilms of *Candida albicans* and *C. dubliniensis* biofilms. The fatty acids were used at 500 μ M and biofilms were incubated at 37 $^{\circ}$ C for 48 h. The mitochondrial metabolism was determined by the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) by mitochondrial enzymes.

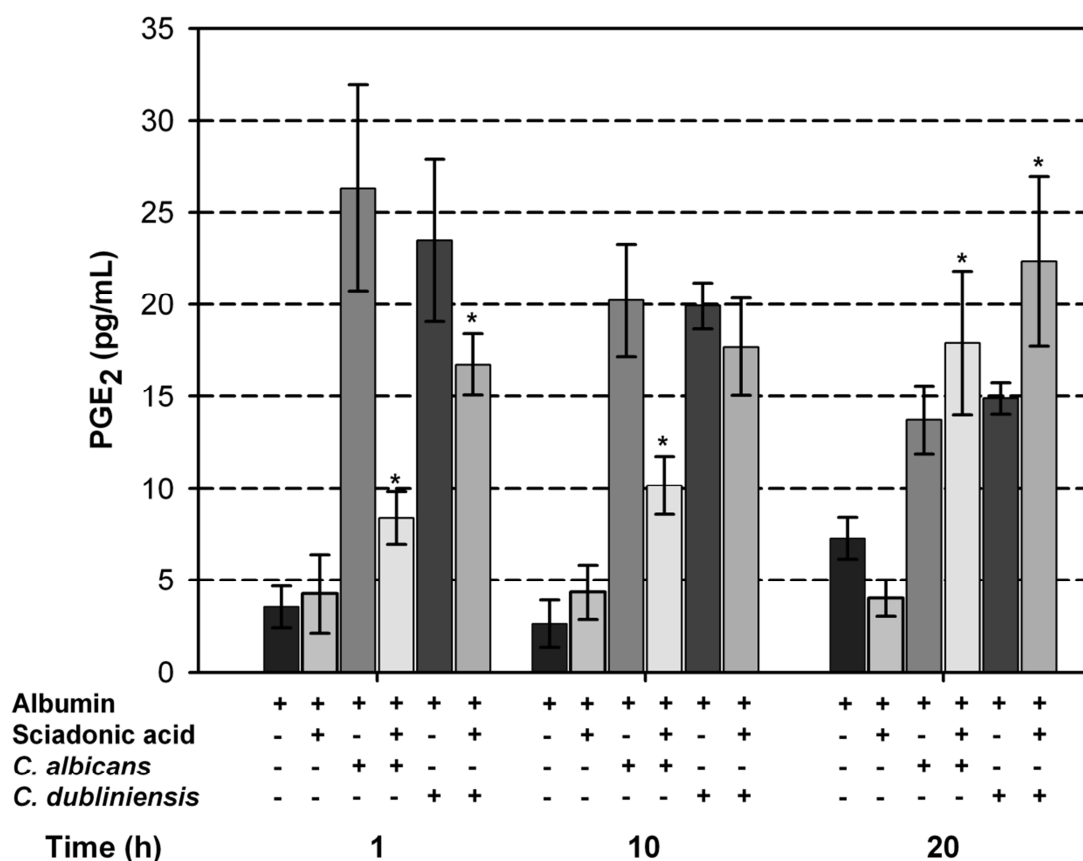


Figure 9. The production of PGE₂ by Hep2C cells after supplementation with albumin or 50 μM sciadonic acid followed by infection with either *C. albicans* NRRL Y-27077 or *C. dubliniensis* NRRL Y-17841T for a maximum of 20 h at 37°C. PGE₂ production was determined by the use of a monoclonal PGE₂ EIA kit (Cayman Chemicals). Values are the mean of duplicate experiments with each sample assayed at two dilutions and each dilution assayed in triplicate and the standard deviation indicated.* Significantly different from unsupplemented infected Hep2C cells ($P \leq 0.05$).

3.4.4. Incorporation of sciadonic acid influences cytokine production by Hep2C cells in response to *C. albicans* and *C. dubliniensis*

We evaluated the production of several different cytokines produced by Hep2C epithelial cells in response to a *Candida* infection. Our data indicated low concentrations of the cytokines produced, suggesting that these epithelial cells are

not primarily responsible for cytokine production in response to *Candida* infection (Steele & Fidel, 2002).

We found that the pro-inflammatory cytokine, IL-6, which acts on different cells and has pleiotropic functions involved in immune responses, inflammation and hematopoiesis (Hirano, 1998), was significantly affected. Our data indicated that epithelial cells did not produce the pro-inflammatory cytokine IL-6 in response to *C. albicans*, during the first hour of infection, but only after 10 h of infection (Figure 10a). In contrast to this, in response to *C. dubliniensis*, epithelial cells produced IL-6 from as early as 1 h of infection. The production of IL-6 during *Candida* infection is in accordance with results obtained by Mostefaoui and co-workers (2004) and Schaller and co-workers (2005), but contrasts findings of Steele and Fidel (2002), who observed that oral and vaginal epithelial cells produced no IL-6 in response to *C. albicans*.

Interestingly, we found that SA supplemented epithelial cells produced significantly less IL-6 in response to the *Candida* species after 10 h and 20 h of infection, i.e. a decrease in the Th2-type response. IL-6 production by epithelial cells in response to *C. albicans*, decreased from 8.0 (\pm 0.7) pg/mL to 3.6 (\pm 3.2) pg/mL and from 7.0 (\pm 0.2) pg/mL to 4.8 (\pm 1.0) pg/mL after 10 h and 20 h, respectively. In response to *C. dubliniensis*, IL-6 production decreased from 8.6 (\pm 0.2) pg/mL to 4.1 (\pm 0.1) pg/mL after 10 h and from 5.4 (\pm 1.1) pg/mL to 3.9 (\pm 0.5) pg/mL after 20 h infection. Similar results were obtained when macrophages supplemented with SA were stimulated with LPS (Huang et al., 2011). This may be beneficial to the host, since Th1-type responses provide resistance to an infection by activating phagocytic cells, whereas Th2-type responses are unable to clear a *Candida* infection, inhibit the development of Th1-type responses and deactivate phagocytic effector cells (Romani, 2000). In addition, the overproduction of IL-6 may lead to the development of chronic autoimmune and inflammatory diseases (Graeve et al., 1993; Ishihara & Hirano, 2002). The decrease in IL-6 production may be due to the decrease in PGE₂ production in the supplemented epithelial cells. In animal models with chronic inflammation, PGE₂ stimulates IL-6 production (Hinson et al., 1996) and Portanova and co-workers (1996) indicated that neutralizing PGE₂ production *in vivo*, not only inhibits inflammation, but also IL-6 production. Interestingly, osteoblasts were also

found to produce PGE₂ in response to IL-6 (Assier et al., 2010), suggesting that IL-6 and PGE₂ production are closely regulated by one another.

IL-6 is also one of the cytokines responsible for inducing the newly identified Th17 cells, to produce pro-inflammatory IL-17 during infection (Bettelli et al., 2007; van der Meer et al., 2010; Weaver et al., 2007). It was also found that *C. albicans* PGE₂ stimulates the production of these Th17 responses in human peripheral blood mononuclear cells by enhancing IL-17 and IL-22 production (Smeekens et al., 2010). Even though it is known that Th17 responses protect the host against *Candida* infections, this was not found in all infection models (Cheng et al., 2010; Conti & Gaffen, 2010; Vautier et al., 2010). Additionally, in these different models, Th17 responses may be more harmful, promoting inflammation and susceptibility to pathogens (Zelante et al., 2007), than providing any benefit, suggesting that there is a variation in *Candida* immunity at different mucosal sites (Gaffen et al., 2011).

In contrast to IL-6, another Th2-type cytokine, IL-13 was produced by unsupplemented cells from 1 h in response to *C. albicans* but not to *C. dubliniensis* (Figure 10b). Sciadonic acid supplementation resulted in a significant increase in IL-13 production in response to both *Candida* species after 1 h of infection. In response to *C. albicans*, IL-13 production increased from 4.0 (\pm 1.6) pg/mL to 13.6 (\pm 3.3) pg/mL and in response to *C. dubliniensis* from 0 to 3.6 (\pm 1.1) pg/mL. However, no significant differences could be observed after 10 h and 20 h of infection. Interleukin-13 is another cytokine with pleiotropic effects, including anti-inflammatory effects, and is responsible for alternative macrophage activation, increasing their ability to clear *C. albicans* infections (Coste et al., 2008). This was also seen in human mononuclear phagocytes where IL-13 minimized *C. albicans* proliferation and tissue penetration by increasing phagocytosis of especially the yeast form of *C. albicans* (Katsifa et al., 2001). This would suggest that the observed increase in IL-13 during the first hour of incubation in the supplemented epithelial cells might increase clearance of *C. albicans* and *C. dubliniensis* infections.

No significant difference was found for the chemotactic cytokine, IL-8, produced in response to both *Candida* species in the supplemented epithelial cells (data not shown). However, the other chemotactic cytokine, monocyte chemoattractant protein 1 (MCP-1), was produced in considerable amounts by the

epithelial cells (Figure 10c), and displayed a significant increase in SA supplemented cells, from 19.2 (\pm 9.4) pg/mL to 41.3 (\pm 7.9) pg/mL compared to unsupplemented cells in response to *C. albicans* after 10 h of infection. This was followed by a decrease after 20 h from 52.6 (\pm 6.9) pg/mL to 41.1 (\pm 3.3) pg/mL in response to *C. albicans* in the supplemented epithelial cells. Sciadonic acid supplementation did not influence MCP-1 production in response to *C. dubliniensis*.

Interestingly, in response to *C. albicans*, the pro-inflammatory cytokine, TNF α , was produced only after 20 h of infection (Figure 10d). This differs from *C. dubliniensis* which stimulated the production of TNF α from 1 h of infection. However, SA supplementation did not significantly influence the production of TNF α in response to *Candida* species. In addition, the other cytokines that were tested for were below the detection limits.

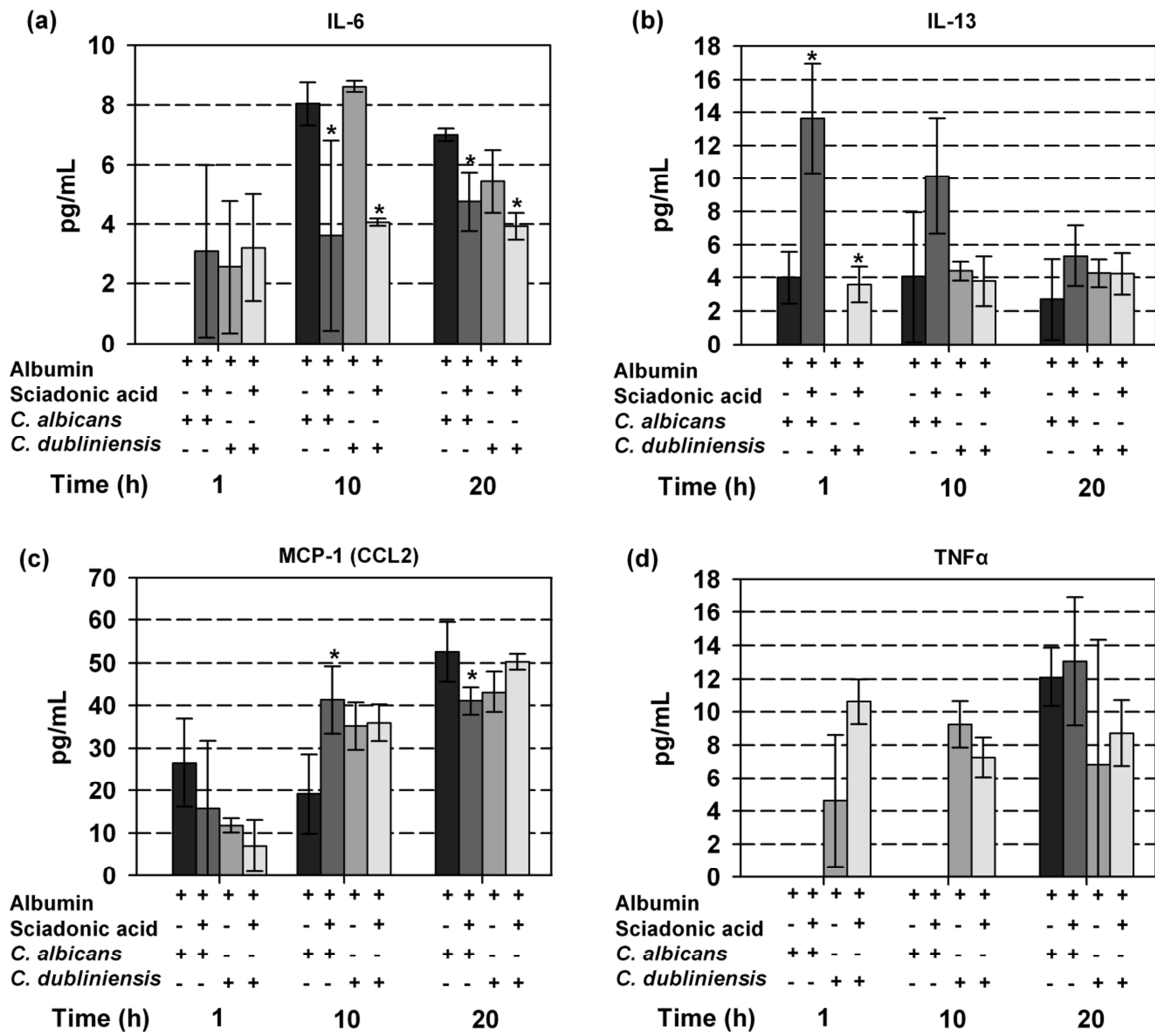


Figure 10. The production of cytokines by confluent Hep2C cells supplemented and unsupplemented, followed by infection with *Candida* spp. incubated for maximum of 20 h at 37°C. The cytokines produced were measured by a Quantibody[®] Human Inflammation Array. (a) Interleukin 6 (IL-6) response of supplemented and unsupplemented Hep2C cells infected with *C. albicans* or *C. dubliniensis*. (b) Interleukin 13 (IL-13) response of supplemented and unsupplemented Hep2C cells infected with *C. albicans* or *C. dubliniensis*. (c) Monocyte chemotactic protein-1 (MCP-1) [chemokine (c-c motif) ligand 2] response of supplemented and unsupplemented Hep2C cells infected with *C. albicans* or *C. dubliniensis*. (d) Tumour necrosis factor-alpha (TNF α) response of supplemented and unsupplemented Hep2C cells infected with *C. albicans* or *C. dubliniensis*. Values are the mean of duplicate experiments with each cytokine arrayed in quadruplicate per slide and the standard deviation indicated. * Significantly different from unsupplemented infected Hep2C cells ($P \leq 0.05$).

3.5. Conclusions

From the data obtained and known literature it is clear that, during host-pathogen interactions, the presence of host and/or fungal prostaglandins can influence the type of immune response and that it could even benefit the pathogen. Recently n-3 PUFAs, such as DHA and EPA, have received significant attention due to their beneficial health effects (Fritsche, 2007; Lands, 2005; Simopoulos, 2002). These fatty acids or their metabolites have been shown to have immunomodulatory and anti-inflammatory activities. However, this can be detrimental to the host, causing a decrease in infectious disease resistance, suggesting that n-3 PUFA consumption can weaken host resistance to a number of pathogens (Anderson & Fritsche, 2002). We found that incorporation of a n-6 non-methylene interrupted PUFA, SA, did not increase DHA or EPA levels, suggesting that it would not lead to an increase in anti-inflammatory prostaglandins and a resultant loss of resistance.

Finally we can conclude that the incorporation of SA (n-6 NMIFAs) into host epithelial phospholipids, leads to a reduction in PGE₂ production and might reduce inflammatory diseases and enhance clearing of the infection. In addition, the inhibition of PGE₂ production may be important in controlling colonization and infection. The approach of changing the lipid composition in susceptible hosts is very feasible and can be done by increasing these fatty acids in their dietary consumption (German et al., 1995; Lopez-Huertas, 2010). However, this still needs to be evaluated *in vivo*.

The use of NanoSAM in our investigation led to the identification of the possible structure of the nodules formed on epithelial cells supplemented with fatty acid. The nodules were proposed to be the fatty acid, as an albumin complex, adhering to the epithelial cells and not outgrowths or blebs of the cell membranes. This investigation expanded the biological application of NanoSAM to human cells. The use of this nanotechnology-based microscopic technique will have a great impact in the field of medicinal research in the future. This can be applied to study drug delivery by nanoparticles to specific cells such as cancer cells.

3.6. Acknowledgements

I would like to thank the personnel from the South African National Control Laboratory for Biological Products at the University of the Free State, Bloemfontein, for providing and assistance with Hep2C cell cultures. I thank Prof. A. Hugo, from the department of Microbial Biochemical and Food Biotechnology for assisting with lipid separation and GC analysis, Prof. P. W. J. van Wyk from the Centre for Microscopy at the University of the Free State, Bloemfontein for assisting with the SEM analysis, Prof. H. C. Swart and Dr. E. Coetsee from the department of Physics at the University of the Free State, Bloemfontein, for the NanoSAM analysis.

I thank the National Research Foundation, South Africa, for funding under the Thuthuka programme (Grant number TTK2007041000014) and the Blue Skies programme (Grant number BS2008092300002).

3.7. References

- Anderson M & Fritsche KL. (2002). (n-3) Fatty acids and infectious disease resistance. *J Nutr* 132: 3566-3576.
- Aronoff DM, Canetti C & Peters-Golden M. (2004). Prostaglandin E₂ inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J Immunol* 173: 559-565.
- Ashman RB, Vijayan D & Wells CA. (2011). IL-12 and related cytokines: function and regulatory implications in *Candida albicans* infection. *Clin Develop Immunol* doi: 10.1155/2011/1686597.
- Assier E, Boissier MC & Dayer JM. (2010). Interleukin-6: From identification of the cytokine to development of targeted treatments. *Joint Bone Spine* 77: 532-536.
- Bagga D, Wang L, Farias-Eisner R, Glaspy JA & Reddy ST. (2003). Differential effects of prostaglandin derived from n-6 and n-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *P Natl Acad Sci* 100: 1751-1756.

- Bahri R, Saidane-Mosbahi D & Rouabhia A. (2010). *Candida famata* modulates Toll-like receptor, β -defensin, and proinflammatory cytokine expression by normal human epithelial cells. *J Cell Physiol* 222: 209-218.
- Barros LF, Kanaseki T, Sabirov R, Morishima S, Castro J, Bittner CX, Maeno E, Ando-Akatsuka Y & Okada Y. (2003). Apoptotic and necrotic blebs in epithelial cells display similar neck diameters but different kinase dependency. *Cell Death Differ* 10: 687-697.
- Berger A & Jomard A. (2001). Fatty acid uninterrupted by a methylene as anti-inflammatory agents in superficial tissues of mammals. Patent no. US 6,280,755 B1.
- Berger A, Monnard I, Baur M, Charbonnet C, Safonova I, Jomard A. (2002). Epidermal anti-inflammatory properties of 5,11,14 20:3: Effects on mouse ear edema, PGE₂ levels in cultured keratinocytes, and PPAR activation. *Lipids Health Dis* 1: 1-12.
- Bettelli E, Korn T & Kuchroo VK. (2007). Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol* 19: 652-657.
- Betz M & Fox BS. (1991). Prostaglandin E₂ inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol* 146: 108-113.
- Bossio DA & Scow KM. (1998). Impacts of carbon and flooding on soil microbial communities: phospholipid fatty acid profiles and substrate utilization patterns. *Microb Ecol* 35: 265-278.
- Castro M, Ralston NVC, Morgenthaler TI, Rohrbach MS & Limper AH. (1994). *Candida albicans* stimulates arachidonic acid liberation from alveolar macrophages through α -mannan and β -glucan cell wall components. *Infect Immun* 62: 3138-3145.
- Chen Q, Ghilardi N, Wang H, Baker T, Xie MH, Gurney A, Grewal IS & de Sauvage FJ. (2000). Development of Th1-type immune responses requires the type I cytokine receptor TCCR. *Nature* 407: 916-920.

- Cheng SC, van de Veerdonk F, Smeekens S, Joosten LAB, van der Meer JWM, Kullberg BJ & Netea MG. (2010). *Candida albicans* dampens host defense by downregulating IL-17 production. *J Immunol* 185: 2450-2457.
- Choi J-K, Ho J, Curry S, Qin D, Bittman R & Hamilton JA. (2002). Interactions of very long-chain saturated fatty acids with serum albumin. *J Lipid Res* 43: 1000-1010.
- Chuang LT, Tsai PJ, Lee CL & Huang YS. (2009). Uptake and incorporation of pinoleic acid reduces n-6 polyunsaturated fatty acid and downstream prostaglandin formation in murine macrophage. *Lipids* 44: 217-224.
- Ciccoli R, Sahi S, Singh S, Prakash H, Zafiriou MP, Ishdorj G, Kock JLF & Nigam S. (2005). Oxygenation by COX-2 (cyclo-oxygenase-2) of 3-HETE (3-hydroxyeicosatetraenoic acid), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3- hydroxyeicosanoids. *J Biochem* 390: 737-747.
- Conti HR & Gaffen SL. (2010). Host responses to *Candida albicans*: Th17 cells and mucosal candidiasis. *Microb Infect* 12: 518-527.
- Coste A, Lagane C, Filipe C, Authier H, Galès A, Bernad J, Douin-Echinard V, Lepert JC, Balard P, Linas MD, Arnal JF, Auwerx J & Pipy B. (2008). IL-13 attenuates gastrointestinal candidiasis in normal and immunodeficient RAG-2^{-/-} mice via peroxisome Proliferator-Activated Receptor- γ activation. *J Immunol* 180: 4939-4947.
- Culp BR, Titus BG & Lands WEM. (1979). Inhibition of prostaglandin biosynthesis by eicosapentaenoic acid. *Prostaglandins Med* 3: 269-278.
- Curry S, Brick P & Franks NP. (1999). Fatty acid binding to human serum albumin: new insights from crystallographic studies. *Biochim Biophys Acta* 1441: 131-140.
- Dalle F, Wächtler B, L'Ollivier C, Holland G, Bannert N, Wilson D, Labruère C, Bonnin A & Hube B. (2010). Cellular interactions of *Candida albicans* with human oral epithelial cells and enterocytes. *Cell Microbiol* 12: 248-271.

- da Silva GM, da Silveira FRX & de Fátima Costa Pires M. (2007). Adherence to HeLa cells, typing by killer toxins and susceptibility to antifungal agents of *Candida dubliniensis* strains. *Braz Oral Res* 21: 87-91.
- Deva R, Ciccoli R, Kock JLF & Nigam S. (2001). Involvement of aspirin-sensitive oxylipins in vulvovaginal candidiasis. *FEMS Microbiol Lett* 198: 37-43.
- Deva R, Shankaranarayanan P, Ciccoli R & Nigam S. (2003). *Candida albicans* induces selectively transcriptional activation of cyclooxygenase-2 in HeLa cells: Pivotal roles of toll-like receptors, p38 mitogen-activated protein kinase, and NF- κ B. *J Immunol* 171: 3047-3055.
- Diaz MT, Alvarez I, De La Fuente J, Sanudo C, Campo MM, Oliver MA, Font i Furnols M, Montossi F, San Julian R, Nute GR & Caneque V. (2005). Fatty acid composition of meat from typical lamb production systems of Spain, United Kingdom, Germany and Uruguay. *Meat Sci* 71: 256-263.
- Dongari-Bagtzoglou A & Fidel PL Jr. (2005). The host cytokine responses and protective immunity in oropharyngeal candidiasis. *J Dent Res* 84: 966-977.
- Ells R, Kock JLF & Pohl CH. (2011). *Candida albicans* or *Candida dubliniensis*? *Mycoses* 54: 1-16.
- Endo Y, Tsunokake K & Ikeda I. (2009). Effects of non-methylene-interrupted polyunsaturated fatty acid, sciadonic (all-cis-5,11,14-eicosatrienoic acid) on lipid metabolism in rats. *Biosci Biotechnol Biochem* 73: 577-581.
- Erb-Downward J & Noverr MC. (2007). Characterisation of prostaglandin E₂ production by *Candida albicans*. *Infect Immun* 75: 3498-3505.
- Filler SG, Ibe BO, Ibrahim AS, Ghannoum MA, Raj JU & Edwards JE. (1994). Mechanisms by which *Candida albicans* induces endothelial cell prostaglandin synthesis. *Infect Immun* 62: 1064-1069.
- Filler SG & Sheppard DC. (2006). Fungal invasion of normally non-phagocytic host cells. *PLoS Pathog* 2: e129.

- Fritsche K. (2007). Important differences exist in the dose-response relationship between diet and immune cell fatty acids in humans and rodents. *Lipids* 42: 961-979.
- Folch J, Lees M & Stanley GHS. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226: 497-509.
- Gaffen SL, Hernández-Santos N & Peterson AC. (2011). IL-17 signaling in host defense against *Candida albicans*. *Immunol Res* 50: 181-187.
- German JB, Gershwin ME & Berger A. (1995). Non-methylene interrupted fatty acids as immunomodulators. Patent no. 5,456,912.
- Goodnight SH Jr, Harris WS, Connor WE & Illingworth DR. (1982). Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. *Arterioscler Thromb Vasc Biol* 2: 87-113.
- Graeve L, Baumann M & Heinrich PC. (1993). Interleukin-6 in autoimmune diseases: Role of IL-6 in physiology and pathology of the immune defense. *Mol Med* 71: 664-671.
- Harizi H & Gualde N. (2006). Pivotal role of PGE₂ and IL-10 in the cross-regulation of dendritic cell-derived inflammatory mediators. *Cell Mol Immunol* 3: 271-277.
- Hedges SR, Agace WW & Svanborg C. (1995). Epithelial cytokine responses and mucosal cytokine networks. *Trends Microbiol* 3: 266-270.
- Hinson RM, Williams JA & Shacter E. (1996). Elevated interleukin-6 is induced by prostaglandin E₂ in a murine model of inflammation: Possible role of cyclooxygenase-2. *Proc Natl Acad Sci* 93: 4885-4890.
- Hirano T. (1998). Interleukin 6 and its receptor: Ten years later. *Intern Rev Immunol* 16: 249-284.
- Huang Y-S, Huang W-C, Li C-W & Chuang L-T. (2011). Eicosadienoic acid differentially modulates production of pro-inflammatory modulators in murine macrophages. *Mol Cell Biochem* doi: 10.1007/s11010-011-0924-0.

- Hur SJ, Ye BW, Lee JL, Ha YL, Park GB & Joo ST. (2004). Effects of conjugated linoleic acid on color and lipid oxidation of beef patties during cold storage. *Meat Sci* 66: 771-775.
- Ishihara K & Hirano T. (2002). IL-6 in autoimmune disease and chronic inflammatory proliferative disease. *Cytokine Growth Factor Rev* 13: 357-368.
- Isseroff RR, Ziboh VA, Chapkin RS & Martinez DT. (1987). Conversion of linoleic acid into arachidonic acid by cultured murine and human keratinocytes. *J Lipid Res* 28: 1342-1349.
- Kalinski P, Hilkens CM, Snijders A, Snijdewint FG & Kapsenberg ML. (1997). IL-12-deficient dendritic cells, generated in the presence of prostaglandin E₂, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 159: 28-35.
- Kalo-Klein A & Witkin SS. (1990). Prostaglandin E₂ enhances and gamma interferon inhibits germ tube formation in *Candida albicans*. *Infect Immun* 58: 260-262.
- Kapoor R & Huang YS. (2006). Gamma linolenic acid: An anti-inflammatory omega-6 fatty acid. *Curr Pharm Biotech* 7: 531-534.
- Katsifa H, Tsaparidou S, Diza E, Gil-Lamaignere C, Walsh TJ & Roilides E. (2001). Effects of interleukin-13 on antifungal activity of human monocytes against *Candida albicans*. *FEMS Immunol. Med Microbiol* 31: 211-217.
- Lacroix M. (2008). Persistent use of “false” cell lines. *Int J Cancer* 122: 1-4.
- Lands WEM. (2005). Dietary Fat and Health: The evidence and the politics of prevention careful use of dietary fats can improve life and prevent disease. *Ann N Y Acad Sci* 1055: 179-192.
- Lim CSY, Rosli R, Seow H-F & Chong P-P. (2011). Transcriptome profiling of endothelial cells during infections with high and low densities of *C. albicans*. *IJMM* 301: 536-546.

- Lopez-Huertas E. (2010). Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. *Pharmacol Res* 61: 200-207.
- Moore AE, Sabachewsky L & Toolan HW. (1955). Culture characteristics of four permanent lines of human cancer cells. *Cancer Res* 15: 598-602.
- Moreno-Ruiz E, Galán-Díez M, Zhu W, Fernández-Ruiz E, d'Enfert C, Filler SG, Cossart P & Veiga E. (2009). *Candida albicans* internalization by host cells is mediated by a clathrin-dependent mechanism. *Cell Microbiol* 11: 1179-1189.
- Mostefaoui Y, Bart C, Frenette M & Rouabhia M. (2004). *Candida albicans* and *Streptococcus salivarius* modulate IL-6, IL-8 and TNF-alpha expression and secretion by engineered human oral mucosa cells. *Cell Microbiol* 6: 1085-1096.
- Noverr MC, Phare SM, Toews GB, Coffey MJ & Huffnagle GB. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* 69: 2957-2963.
- Noverr MC, Toews GB & Huffnagle GB. (2002). Production of prostaglandins and leukotrienes by pathogenic fungi. *Infect Immun* 70: 400-402.
- Petitpas I, Grüne T, Bhattacharya AA & Curry S. (2001). Crystal structures of human serum albumin complexed with monounsaturated and polyunsaturated fatty acids. *J Mol Biol* 314: 955-960.
- Pfaller MA & Diekema DJ. (2010). Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol* 36: 1-53.
- Phadungath C. (2005). Casein micelle structure: a concise review. *J Sci Technol* 27: 201-212.
- Phan QT, Myers CL, Fu Y, Sheppard DC, Yeaman MR, Welch WH, Ibrahim AS, Edwards JE Jr & Filler SG. (2007). Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biology* 5: 543-557.

- Piganelli JD & Mathews CE. (2007). Autoreactive T-cell responses: new technology in pursuit of an old nemesis. *Pediatr Diabetes* 8: 249-251.
- Portanova JP, Zhang Y, Anderson GD, Hauser SD, Masferrer JL, Seibert K, Gregory SA & Isakson PC. (1996). Selective neutralization of prostaglandin E₂ blocks inflammation, hyperalgesia, and interleukin 6 production *in vivo*. *J Exp Med* 184: 883-891.
- Ramage G, Saviile SP, Thomas DK & Lopez-Ribot JL. (2005). *Candida* biofilms: An update. *Eukaryot Cell* 4: 633-638.
- Romani L. (2000). Innate and adaptive immunity in *Candida albicans* infections and saprophytism. *J Leukoc Biol* 68: 175-179.
- Rouabhia M, Ross G, Pagé N & Chakir J. (2002). Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation. *Infect Immun* 70: 7073-7080.
- Schaller M, Korting HC, Borelli C, Hamm G & Hube B. (2005). *Candida albicans*-secreted aspartic proteinase modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis. *Infect Immun* 73: 2758-2765.
- Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR, Mirick G & Moussignac RL. (2002). Resolvins: A family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med* 196: 1025-1037.
- Shibata Y, Henriksen RA, Honda I, Nakamura RM & Myrvik QN. (2005). Splenic PGE₂-releasing macrophages regulate Th1 and Th2 immune responses in mice treated with heat-killed BCG. *J Leukoc Biol* 78: 1281-1290.
- Simopoulos AP. (2002). Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* 21: 495-505.
- Slover HT & Lanza E. (1979). Quantitative analysis of food fatty acids by capillary gas chromatography. *J Am Oil Chem Soc* 56: 933-943.

- Smeekens SP, van de Veerdonk FL, van der Meer JWM, Kullberg BJ, Joosten LAB & Netea MG. (2010). The *Candida* Th17 response is dependent on mannan- and β -glucan-induced prostaglandin E₂. *Int Immunol* 22: 889-895.
- Smith WL. (1989). The eicosanoids and their biochemical mechanisms of action. *Biochem J* 259: 315-324.
- Snijdewint FGM, Kalinski P, Wierenga EA, Bos JD & Kapsenberg M. (1993). Prostaglandin E₂, differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J Immunol* 150: 5321-5329.
- Southern P, Horbul J, Maher D & Davis DA. (2008). *C. albicans* colonization of human mucosal surfaces. *PLoS One* 3: e2067.
- Spector AA. (1975). Fatty acid binding to plasma albumin. *J Lipid Res* 16: 165-179.
- Stadnyk AW. (1994). Cytokine production by epithelial cells. *FASEB J* 8: 1041-1047.
- Steele C & Fidel PL Jr. (2002). Cytokine and chemokine production by human oral and vaginal epithelial cells in response to *Candida albicans*. *Infect Immun* 70: 577-583.
- Sullivan DJ, Moran GP, Pinjon E, Al-Mosaid A, Stokes C, Vaughan C & Coleman DC. (2004). Comparison of the epidemiology, drug resistance mechanisms and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res* 4: 369-376.
- Sullivan DJ, Westerneng TJ, Haynes KA, Bennett DE & Coleman DC. (1995). *Candida dubliniensis* sp. nov.: phenotypic and molecular characterisation of a novel species associated with oral candidosis in HIV infected individuals. *Microbiology* 141: 1507-1521.
- Suram S, Gangelhoff TA, Taylor PR, Rosas M, Brown GD, Bonventre JV, Akira S, Uematsu S, Williams DL, Murphy RC & Leslie CC. (2010). Pathways regulating cytosolic phospholipases A₂ activation and eicosanoid production in macrophages by *Candida albicans*. *J Biol Chem* 285: 30676-30685.

- Swart CW, Swart HC, Coetsee E, Pohl CH, van Wyk PWJ & Kock JLF. (2010). 3-D architecture and elemental composition of fluconazole treated yeast asci. *Sci Res Essays* 5: 3411-3417.
- Tanaka T, Morishige J, Iwawaki D, Fukuhara T, Hamamura N, Hirano K, Osumi T & Satouchi K. (2007). Metabolic pathway that produces essential fatty acids from polymethylene-interrupted polyunsaturated fatty acids in animal cells. *FEBS J* 274: 2728-2737.
- Tanaka T, Morishige J, Takimoto T, Takai Y & Satouchi K. (2001). Metabolic characterization of sciadonic acid as an effective substitute for arachidonate of phosphatidylinositol. *Eur J Biochem* 268: 4928-4939.
- Tanaka T, Takimoto T, Morishige J, Kikuta Y, Sugiura T & Satouchi K. (1999). Non-methylene-interrupted polyunsaturated fatty acids: effective substitute for arachidonate of phosphatidylinositol. *Biochim Biophys Res Commun* 264: 683-688.
- Trang LE. (1980). Prostaglandins and inflammation. *Sem Arth Rheum* 9: 153-159.
- Tsarfaty I, Sandovsky-Losica H, Mittelman L, Berdicevsky I & Segal E. (2000). Cellular actin is affected by interaction with *Candida albicans*. *FEMS Microbiol Lett* 189: 225-232.
- van der Meer JWM, van de Veerdonk FL, Joosten LAB, Kullberg BJ & Netea MG. (2010). Severe *Candida* spp. infections: new insights into natural immunity. *Int J Antimicrob Ag* S36: S58-S62.
- van der Pouw Kraan TCTM, Boeije LCM, Smeenk RJT, Wijdenes J & Aarden LA. (1995). Prostaglandin-E₂ is a potent inhibitor of human interleukin-12 production. *J Exp Med* 181: 775-779.
- Vautier S, da Glória Sousa M & Brown GD. (2010). C-type lectins, fungi and Th17 responses. *Cytokine Growth Factor Rev* 21: 405-412.

- Villar CC, Kashleva H & Dongari-Bagtzoglou A. (2004). Role of *Candida albicans* polymorphism in interactions with oral epithelial cells. *Oral Microbiol Immunol* 19: 262-269.
- Weaver CT, Hatton RD, Mangan PR & Harrington LE. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25: 821-852.
- Weissenbacher TM, Witkin SS, Gangelmaier A, Scholz C, Friese K & Mylonas I. (2009). Relationship between recurrent vulvovaginal candidosis and immune mediators in vaginal fluid. *Eur J Obstet Gynaecol Reprod Biol* 144: 59-63.
- Witkin SS, Kalo-Klein A, Galland L, Teich M & Ledger WJ. (1991). Effect of *Candida albicans* plus histamine on prostaglandin E₂ production by peripheral blood mononuclear cells from healthy women and women with recurrent candidal vaginitis. *J Infect Dis* 164: 396-399.
- Yang YL. (2003). Virulence factors of *Candida* species. *J Microbiol Immunol Infect* 36: 223-228.
- Zakikhany K, Naglik JR, Schmidt-Westhausen A, Holland G, Schaller M & Hube B. (2007). *In vivo* transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell Microbiol* 9: 2938-2954.
- Zelante T, De Luca A, Bonifazi P, Montagnoli C, Bozza S, Moretti S, Belladonna ML, Vacca C, Conte C, Mosci P, Bistoni F, Puccetti P, Kastelein RA, Kopf M & Romani L. (2007). IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur J Immunol* 37: 2695-2706.

CHAPTER 4

***Identification of Candida albicans genes
differentially expressed in the presence of
arachidonic and sciadonic acid***

4.1. Abstract

Although *Candida* species, more specifically *C. albicans*, are the most common pathogenic yeasts, little is known about the mechanism involved in eicosanoid production, a virulence factor of these organisms. This is an important area of research which may aid in the understanding of the complex interactions between host and pathogen that may possibly lead to the identification of novel antifungals or drug targets. In this study, expression profiling using *C. albicans* DNA microarrays and a Two-Step Reverse transcriptase-qPCR (RT-qPCR) were used to evaluate the regulation of *C. albicans* genes during incubation in the presence of arachidonic acid (AA) [20:4(n-6)], the major precursor for PGE₂ production, and sciadonic acid. The results indicated that the differentially expressed genes had diverse functions not normally associated with cell growth. Although we found that genes encoding proteins with oxidoreductase and hydrolase activity were regulated, these gene products are not known to be involved in PGE₂ synthesis. However, the ABC transporters, specifically the *SNQ2* gene, were found to be regulated. However, this may be in response to 20-carbon fatty acids rather than to the production of PGE₂. Additionally, no genes involved in biofilm formation were differentially expressed, even though genes involved in filamentous and hyphal growth were expressed. This may be attributed to PGE₂ production from AA. As expected with the addition of polyunsaturated fatty acids, genes involved in response to oxidative stress were affected. In addition, carbohydrate metabolic processes were also affected, suggesting a switch to alternative carbon metabolism such as the glyoxylate cycle and β -oxidation of fatty acids. This work provides insight into the transcriptional responses of *C. albicans* biofilms in the presence of AA. Further study of these differentially expressed genes is needed to evaluate how they may be involved in AA metabolism and PGE₂ production.

4.2. Introduction

Candida species are the 4th most common pathogens responsible for systemic bloodstream infections (Pfaller & Diekema, 2010) with the most frequently isolated, *C. albicans*, contributing to 50% of these cases with high mortality rates (Pappas et al., 2003; Wisplinghoff et al., 2004). Some of these species exist as commensals of mucosal membranes in most healthy individuals and other warm-blooded animals, where they grow without causing any damage (Ramage et al., 2001; Sullivan et al., 2004). However, they are known as opportunistic pathogens since changes in the host, such as immune-compromized or hospitalized individuals, will lead to infections ranging from mild to life threatening.

Eicosanoid production by fungi is seen as an important virulence factor, not only in *C. albicans* but in other fungi as well (e.g. *Aspergillus* spp., *Cryptococcus neoformans*) (Alem & Douglas, 2005; Ciccoli et al., 2005; Erb-Downward & Huffnagle, 2007; Erb-Downward & Noverr, 2007; Noverr et al., 2002; Tsitsigiannis et al., 2005). The production of these fungal eicosanoids from arachidonic acid (AA) [20:4(n-6)], has received a lot of attention during the last decade. Although attempts have been made to elucidate the pathways involved in eicosanoid production by fungi, limited information in this regard is available (Erb-Downward et al., 2008; Erb-Downward & Noverr, 2007; Noverr et al., 2001; Tsitsigiannis et al., 2005). This is an important area of research which may aid in the understanding of the complex interaction between host and pathogen and may possibly lead to the identification of novel antifungals or drug targets.

The completion of the *C. albicans* genome sequencing project (Jones et al., 2004) and the availability of *C. albicans* DNA microarrays enabled genomic hybridization studies of the genome of *C. albicans*, as well as the closely related species, *C. dubliniensis* (Moran et al., 2004), under different environmental conditions. This is also helpful to identify genes involved in virulence in pathogenic fungi. In this study, genomic hybridization studies using *C. albicans* DNA microarrays were used to evaluate the regulation of *C. albicans* genes during incubation in the presence of AA. This was done to try to elucidate a possible pathway involved in prostaglandin production by this species. Genes were selected and validated with a Two-Step Reverse transcriptase-qPCR (RT-qPCR). It is known that a non-methylene

interrupted anti-inflammatory fatty acid sciadonic acid (SA) [20:3(n-6)], that competes with AA and is easily incorporated into the phospholipids of mammalian cells, cannot be metabolized to prostaglandins (Berger et al., 2002; Berger & Jomard, 2001; German et al., 1995; Tanaka et al., 2001). Therefore, it was also of interest to evaluate the effect of this fatty acid on the expression of the selected genes.

4.3. Materials and methods

4.3.1. Strains used

The following strains were used in this study: *Candida albicans* NRRL Y-27077 (isolated from a skin lesion, Germany) and *C. dubliniensis* NRRL Y-17841T (isolated from oral cavity of HIV-infected patient, Dublin, Ireland). Both strains were obtained from the Agricultural Research Service Culture Collection of the United States Department of Agriculture. All strains were maintained on Yeast Malt Extract (YM) agar (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 16 g/L agar) at room temperature.

4.3.2. Biofilm formation

Strains from 24 h old cultures on YM agar plates were inoculated into 20 mL synthetic media (6.7 g/L Yeast Nitrogen Base, 10 g/L glucose) in 50 mL polypropylene conical centrifuge tubes (Becton Dickinson Labware, USA) and incubated at 30°C for 48 h. The cells were harvested by centrifugation (10 min at 4412 x g) (Heraeus, Megafuge 1R), washed twice with phosphate buffered saline (PBS) (OXOID, UK) and resuspended into filter sterilized RPMI-1640 medium (Sigma-Aldrich, USA). The cells were counted and diluted to 1×10^6 cells/mL in 150 mL RPMI-1640 medium containing a final concentration of 500 μ M AA (Sigma Aldrich, USA) or 500 μ M SA (Lipidox, Stockholm, Sweden) (in 0.38% v/v ethanol) (Sigma Aldrich, USA). Appropriate controls were included. These suspensions were dispensed into cell culture plates (Corning, USA) and incubated at 37°C for up to 48 h, to allow biofilm formation (Ramage et al., 2001).

4.3.3. RNA extraction

Biofilms were scraped from the plates after 8, 24 and 48 h and washed three times with PBS at 4°C. Total RNA was extracted immediately after harvesting the cells. To the cell pellet 1 M sorbitol (Sigma Aldrich, RSA), 0.1 M EDTA (Merck, RSA) (pH 7.4), 0.1% (v/v) 2-mercaptoethanol (Sigma, RSA) and lyticase (Sigma, RSA) (final concentration of 50 U per 1×10^7 cells) were added. After mixing, the reactions were incubated at 30°C for 30–60 min with gentle shaking to produce spheroplasts. The spheroplasts were recovered by centrifugation (600 x *g* for 5 min) (Eppendorf Centrifuge 5415 D) at room temperature. Total RNA extraction and purification were done with an RNeasy Mini Kit (Qiagen) with an on-column DNase (RNase-Free DNase Set, Qiagen) digestion step included. The quality of the RNA was determined with formaldehyde gel electrophoresis (Kohrer and Domdey, 1991) and the concentration of RNA spectrophotometrically (NanoDrop ND-1000 Spectrophotometer). An additional DNase digestion followed by a second purification with an RNeasy Mini Kit was performed. Polymerase chain reactions (2720 Thermal cycler, Applied Biosystems) using the primer pair SNQ2-2F (5'–TATGCTGATGCCGTTGTTGG–3') and SNQ2-2R (5'–CAATGGCCCAAGCAGATTG–3'), followed by gel electrophoresis, were carried out to verify the absence of genomic DNA (Figure 1). When no signal is observed, the RNA is free of genomic DNA. Similar results were obtained for all experimental samples. The RNA was stored at -80°C until used.

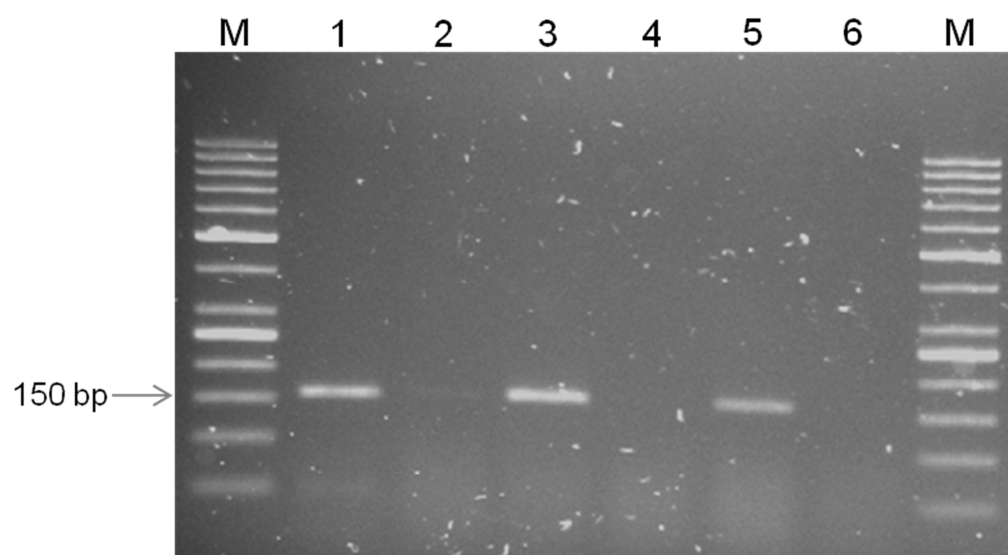


Figure 1. Picture of gel electrophoresis indicating DNase treatment of the RNA template for microarray and RT-qPCR analysis. The primer pair *SNQ2-2F* and *SNQ2-2R* was used to verify the absence of any DNA giving a product of 157 bp. (M) GeneRuler™ 50 bp DNA Ladder (Fermentas). (1) *C. albicans* 8 h control. (2) *C. albicans* 8 h control DNase treated. (3) *C. albicans* 24 h control. (4) *C. albicans* 24 h control DNase treated. (5) *C. albicans* 48 h control. (6) *C. albicans* 48 h control DNase treated.

4.3.4. cDNA synthesis for microarray analysis

Note that only RNA obtained from *C. albicans*, grown in the presence and absence of 500 μ M AA was used for microarray analysis. Each sample was done in triplicate with a dye swap included. The microarray experiment was designed so that the hybridizations obtained had optimal statistical outlay as illustrated in figure 2. Total RNA of each sample was divided into two fractions at a final concentration of 15 μ g/22 μ L. To this 10-20 U RNase inhibitor (Invitrogen, Inqaba Biotech), 0.5 μ g Oligo(dT)₁₂₋₁₈ primer and 0.5 μ g random nonamer primer were added. This suspension was mixed well and incubated at 70°C for 5 min and 10 min at room temperature. To this, 1.3 mM dATP, 1.3 mM dCTP, 1.3 mM dGTP, 0.5 mM dTTP, 0.8 mM aminoallyl-dUTP, 200 U SuperScript® III Reverse Transcriptase, first strand buffer (250 mM Tris-HCl, pH 8.3 at room temperature; 375 mM KCl; 15 mM MgCl₂) and 26.7 mM dithiothreitol (DTT), were added, mixed gently and incubated at 42°C

for 3 h. An additional 200 U SuperScript[®] III Reverse Transcriptase was added and incubated overnight at 42°C. RNA was hydrolyzed by adding 1 M NaOH, 0.5 M EDTA and incubation at 65°C for 15 min. pH was neutralized with 1 M HEPES, pH 7.0. The unincorporated dNTPs and RNA were removed with a RNeasy MinElute Cleanup Kit (Qiagen) and the cDNA concentration determined spectrophotometrically. The cDNA was dried in a SpeediVac (Concetrator plus, Eppendorf, Merck, RSA), and re-suspended in 5 µL NaHCO₃ buffer, pH 9.0.

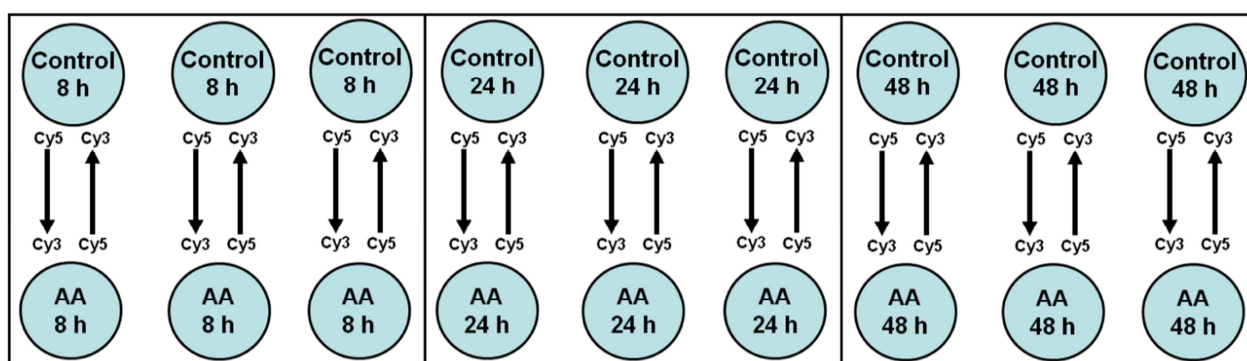


Figure 2. A schematic diagram indicating the outlay of the microarray experiment. The AA treated sample was compared to the untreated control at each time point. Each circle illustrates the sample being hybridized, with the specifics of the samples described in the circle. The arrow indicates one slide, with the arrow end indicating the Cy3 labelled sample, and the blunt end the Cy5 labelled sample. Each sample had three biological replicates and a technical replicate (dye swap) for each biological replicate.

4.3.5. cDNA dye-coupling for microarray analysis

Relevant Cy3- and Cy5-N-hydroxysuccinimide esters (GE Healthcare, UK; CY Dye Post Labelling reactive Dye Pack; Amersham) were added to each re-suspended cDNA sample and incubated at room temperature for 2 h with regular, gentle agitation. Relevant Cy3- and Cy5-N-Hydroxysuccinimide esters were added to each re-suspended cDNA sample and incubated at room temperature for 2 h with regular, gentle agitation. Subsequently 0.1 M sodium acetate, pH 5.2, was added and unincorporated Cy dyes were removed with the RNeasy MinElute Cleanup Kit.

Cy3 and Cy5 labelled samples were combined to 150-200 pmol each and dried down.

4.3.6. Hybridization

Slides containing oligomer based spotted microarrays for the *C. albicans* genome, representing 6346 ORFs of the predicted 6354 ORFs, spotted in triplicate on epoxy slides, were purchased from Washington University School of Medicine, Genome Sequencing Centre, USA (Brown et al., 2006). The pre-hybridization of the epoxy slides was done according to the manufacturer's instructions. Dye labelled samples were reconstituted in hybridization buffer [35% (v/v) formamide, 5x saline sodium citrate, 0.1% (w/v) sodium dodecyl sulfate, 0.1 mg/mL salmon sperm, deionised water], incubated at 95°C for 2 min and 42°C until hybridization. Biological samples were hybridized in triplicate with dye-swap technical replicates included using a direct comparison design (Naidoo et al., 2005), in which the AA treated samples were compared to control samples at each time point (Figure 2). Hybridization was performed overnight at 42°C. Following post-hybridization washes, the slides were dried by centrifugation and scanned with a GenePix 4000B scanner (Axon Instruments Inc., Foster City, CA, USA). Fluorescence intensity values were determined with GenePix Pro 5.1, and data was analysed using limma in the R computing environment (Smyth, 2005). Data was normalized and analyzed according to Crampton and co-workers (2009). Individual linear models were based on the comparison between treated and untreated samples for each time point. The FDR function (Benjamini-Hochberg method) in limma was used to correct for false positives, and genes were classified as differentially expressed if they showed a *P*-value ≤ 0.05 and a fold change ≥ 1.5 .

4.3.7. cDNA synthesis for Reverse transcriptase-qPCR analysis

Please note that both *C. albicans* and *C. dubliniensis* RNA, with 500 μ M AA and SA treatment, were used for RT-qPCR analysis. cDNA synthesis was done using the BioRad iScript Select cDNA synthesis Kit according to the manufacturer's instructions. Briefly, cDNA was synthesized from 500 ng total RNA, primed by

oligo(dT) at 42°C for 90 min, followed by a 5 min incubation step at 85°C, to inactivate the reverse transcriptase. The cDNA product was then stored at -20°C until further analysis.

4.3.8. Reverse transcriptase-qPCR analysis

Gene specific primers were designed with Primer3 software from The Whitehead Institute (Rozen & Skaletsky, 2000). Criteria used were amplified products between 75 and 250 bp with a T_m of between 55-65°C (primer sequences are shown in Table 1). The primers were purchased from Integrated DNA Technologies (IDT) [WhiteSci, Whitehead Scientific (Pty) Ltd, RSA] and resuspended in 1 x TE solution (10 mM Tris, pH 8.0, 0.1 mM EDTA) (IDT, RSA) to a final concentration of 100 μ M. The optimal T_m of the primers was confirmed by gradient PCR (G-STORM PCR machine) with temperatures ranging between 50-60°C. Electrophoretic analysis of the amplification products confirmed that the primers amplified only a single product with expected size (Figure 3).

RT-qPCR reaction mixtures contained 1 μ L of diluted cDNA (1:30), 0.3 μ M of each primer, 5 μ L SsoFast™ EvaGreen Supermix (BioRad, RSA) in a total volume of 10 μ L. During each run, a 1:4 dilution series, as well as a non-template control, were included. The standard was used to generate a 7-point standard curve for data analysis. Reaction mixtures were incubated for 30 seconds at 95°C, followed by 40 amplification cycles of 5 seconds at 95°C, 5 seconds at 59.5°C. A final melt curve between 65-95°C in 0.5°C increments was included.

For all reference and target genes studied, three independent biological replicates of each experimental condition were evaluated in technical triplicates. PCR efficiencies and optimal quantification cycle threshold (Cq values) were estimated using Bio-Rad CFX Manager software to calculate the relative level of expression of the reference genes and target genes for all experimental samples using the standard curve. Expression data of the target genes were normalized against the most stably expressed reference genes using the Biogazelle qBASE plus software (Hellemans et al., 2007). Finally, the fold-change in gene expression was calculated by dividing the normalized value for the experimental sample (treated) by the normalized value for the control sample (untreated).

Table 1: List of primers used for Reverse transcriptase-qPCR.

Gene symbol	Gene size	Amplicon size	Primer sequence (5' – 3')
<i>CaACT1</i>	1789	113	Forward: TGACGACGCTCCAAGAGCTG Reverse: TGGATTGGGCTTCATCACCAACA
<i>CaHST6</i>	3972	118	Forward: TCACCATCAGATTCACCGGCT Reverse: TGGCAATTGCTGAACCACCCA
<i>CaSNQ2</i>	4488	128	Forward: AGCTCGTGATGCTGGCAAGT Reverse: CACCCATGGCATTTCACCG
<i>CaTEF1</i>	1377	146	Forward: ACCACCAACCAGACCAACCG Reverse: CACCAGCTGGGGCGAAAGTA
<i>CaVPS13</i>	9252	215	Forward: TGGTACGTGGCAAGATGCCC Reverse: TGCTTCTGGCTGTTGGGGTG
<i>CdACT1</i>	1131	108	Forward: ACGCTCCAAGAGCTGTGTTCC Reverse: TGGATTGGGCTTCGTCACCG
<i>CdHST6</i>	3951	187	Forward: GGTTTCAGCAATTGCAACGCCA Reverse: GCCACGTGAACATGCCTAGC
<i>CdSNQ2</i>	4485	230	Forward: CGGGCGGTGATACTACTGCT Reverse: ACCTGCACCTGATTCACCCA
<i>CdTEF1</i>	1377	147	Forward: ACCACCAACCAGACCAACCG Reverse: ACACCAGCTGGGGCAAAGT
<i>CdVPS13</i>	9255	186	Forward: TATCCCTAGCGTGGACGGGG Reverse: GGGGGTGGTGCCTCTTGTTT

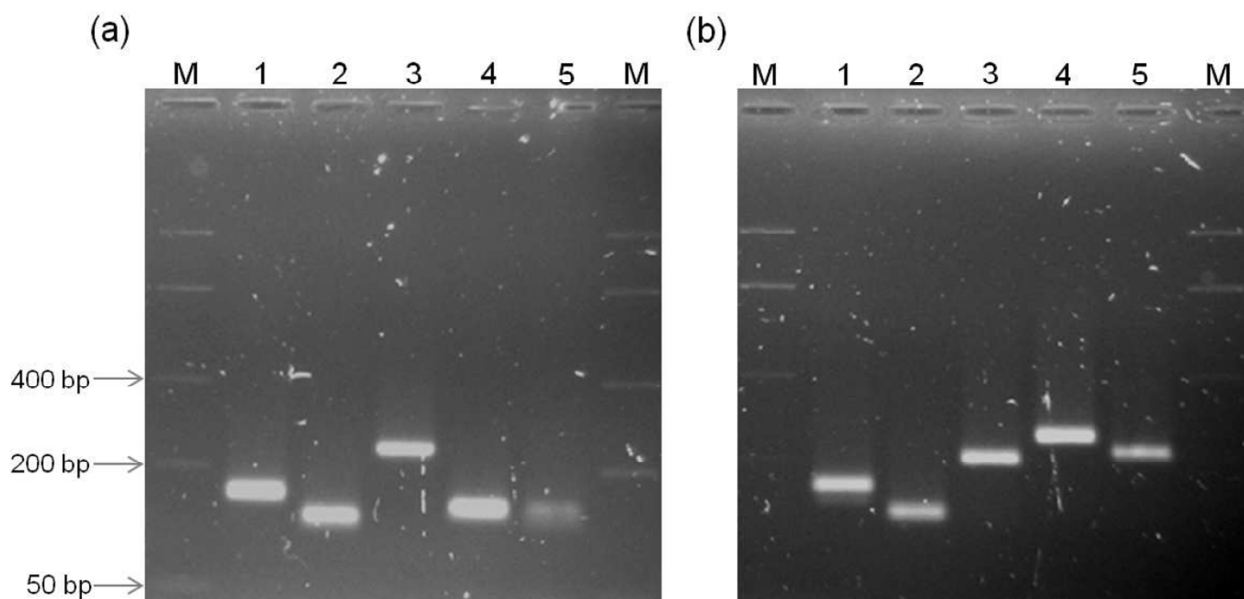


Figure 3. Picture of gel electrophoresis indicating the presence of the expected product sizes used for Reverse transcriptase-qPCR analysis. *Candida albicans* and *C. dubliniensis* cDNA were amplified with the corresponding primers (Table 1). (a) *C. albicans* cDNA, (b) *C. dubliniensis* cDNA. (M) FastRuler™ Low Range DNA Ladder (Fermentas, RSA), (1) *TEF1*. (2) *ACT1*. (3) *VPS13*. (4) *SNQ2*. (5) *HST6*.

4.4. Results and discussion

4.4.1. Microarray analysis

The main objective of this study was to identify genes differentially expressed by *C. albicans* biofilms in the presence of AA. The time points selected represented different developmental phases during biofilm formation i.e. the early phase (8 h), intermediate phase (24 h) and mature phase (48 h) (Chandra et al., 2001). Data obtained indicated that a total of 2.5% of the 6346 genes were differentially expressed at least 1.5 fold ($P \leq 0.05$) after 8 h and 1.3% after 24 and 48 h in the presence of 500 μM AA (Table 2). This is based on genes with a fold change of equal to or more than 1.5 ($P \leq 0.05$). These results indicated that most of the genes are differentially expressed during the early phase of biofilm formation, suggesting that few new metabolic activities are initiated by the more mature biofilm. Similar

results were obtained by Yeater and co-workers (2007) when they compared gene expression between *C. albicans* biofilms and planktonic cells at the different phases.

The number of genes with the same fold change after 8, 24 and 48 h were determined (Figure 4). After 8 h, most differentially expressed genes were down-regulated between 1.5 and 2 fold. After 24 and 48 h, more genes were up-regulated.

Table 2: The % of *Candida albicans* genes regulated during the 48 h incubation period.

	Up-regulated (%)	Down-regulated (%)	Total genes (%)
8 h	0.3	2.2	2.5
24 h	0.7	0.6	1.3
48 h	0.9	0.4	1.3

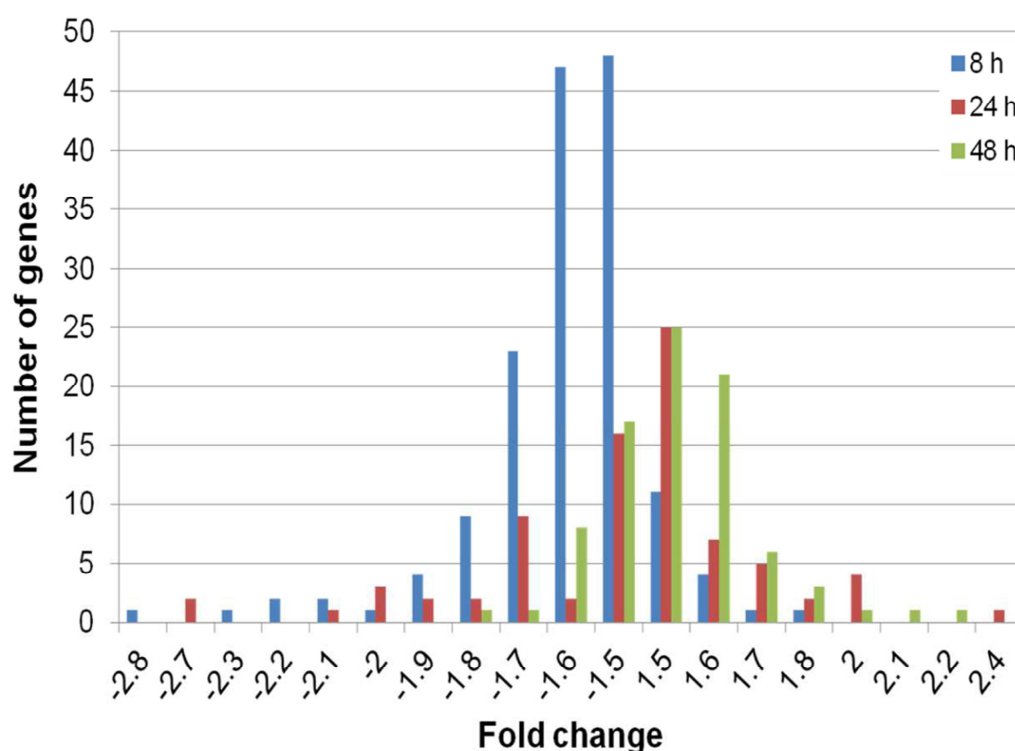


Figure 4. Graph indicating the number of *Candida albicans* genes either up-regulated (fold change ≥ 1.5) or down-regulated (fold change ≤ -1.5) during incubation at 37°C in the presence of 500 μ M arachidonic acid as determined by microarray analysis.

4.4.1.1. *Molecular function and biological processes of regulated genes*

The genes that were significantly regulated were annotated according to their molecular function (Figure 5) and biological processes (Figure 6) using the Gene Ontology Slim Mapper from the *Candida* Genome Database (CGD) (Inglis et al., 2011). The molecular function describes the tasks performed by individual gene products and a biological process is a series of events accomplished by one or more ordered assemblies of molecular functions. During the first 8 h of incubation, 65% of the genes that were significantly up-regulated had an unknown molecular function (Figure 5a). At 24 and 48 h, the genes with unknown molecular function decreased to 35 and 40%, respectively (Figures 5b, c). Among the differentially expressed genes with a known molecular function, most coded for enzymes with hydrolase, oxidoreductase, transferase and transporter activity. Most of these genes are not usually required for normal cell growth (Yeater et al., 2007).

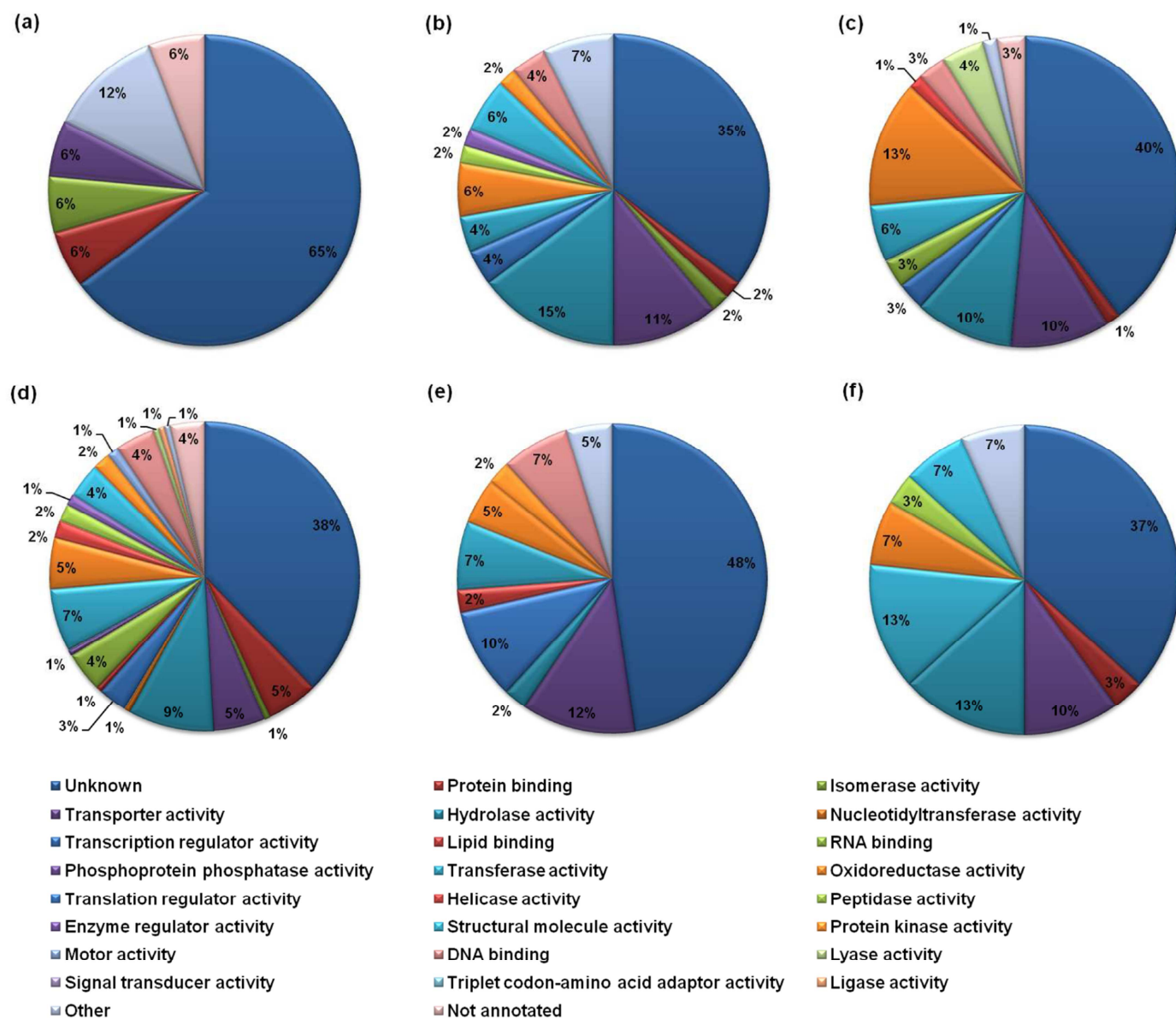


Figure 5. Pie charts indicating the percentage of the frequency of the *Candida albicans* genes, with their corresponding molecular functions according to the Gene Ontology Slim Mapper from the *Candida* Genome Database (CGD), that were differentially expressed during incubation at 37°C in the presence of 500 μM arachidonic acid. Genes up-regulated after (a) 8 h, (b) 24 h, (c) 48 h. Genes down-regulated after (d) 8 h, (e) 24 h, (f) 48 h.

Similar to the molecular functions, most of the biological processes of genes differentially expressed are still unknown (Figure 6). Filamentous growth, organelle organization, response to chemical stimulus, response to stress, RNA metabolic processes and transport were amongst the biological processes regulated in *C. albicans* biofilms in the presence of AA. In addition, no genes involved in biofilm formation were differentially expressed, even though genes involved in filamentous and hyphal growth were differentially expressed. In our study, *HYM1* (orf19.796) which form part of the RAM network (regulation of Ace2p transcription factor and polarized morphogenesis) (Nelson et al., 2003), was up-regulated more than 2-fold up-regulated in the presence of AA during the incubation period (Figure 7). The RAM network, as the name states, regulates polarized morphogenesis through a conserved signalling network and has been well studied in *Saccharomyces cerevisiae* (Nelson et al., 2003). This network consists of six genes, namely *CBK1*, *HYM1*, *KIC1*, *MOB2*, *PAG1* and *SOG2*. In a study by Song and co-workers (2008), it was found that these genes are not necessary for *C. albicans* viability but are important for *C. albicans* hyphal growth. In our study, the only other genes belonging to this network that was significantly regulated were the kinases *CBK1* (orf19.4909) and *KIC1* (orf19.191) which were both down-regulated after 8 and 24 h (Supplementary table). In addition, *SSY1* (orf19.814) (Figure 7) which encodes for an amino acid sensor required for hyphal growth (Brega et al., 2004) was up-regulated more than 2-fold after 24 and 48 h incubation. This can possibly be attributed to the production of PGE₂ from AA (Chapter 2), because it is known that PGE₂ has the ability to stimulate germination in *C. albicans* (Kalo-Klein & Witkin, 1990; Noverr & Huffnagle, 2004). This is in contrast to AA that does not have any effect on *C. albicans* germination (Noverr & Huffnagle, 2004).

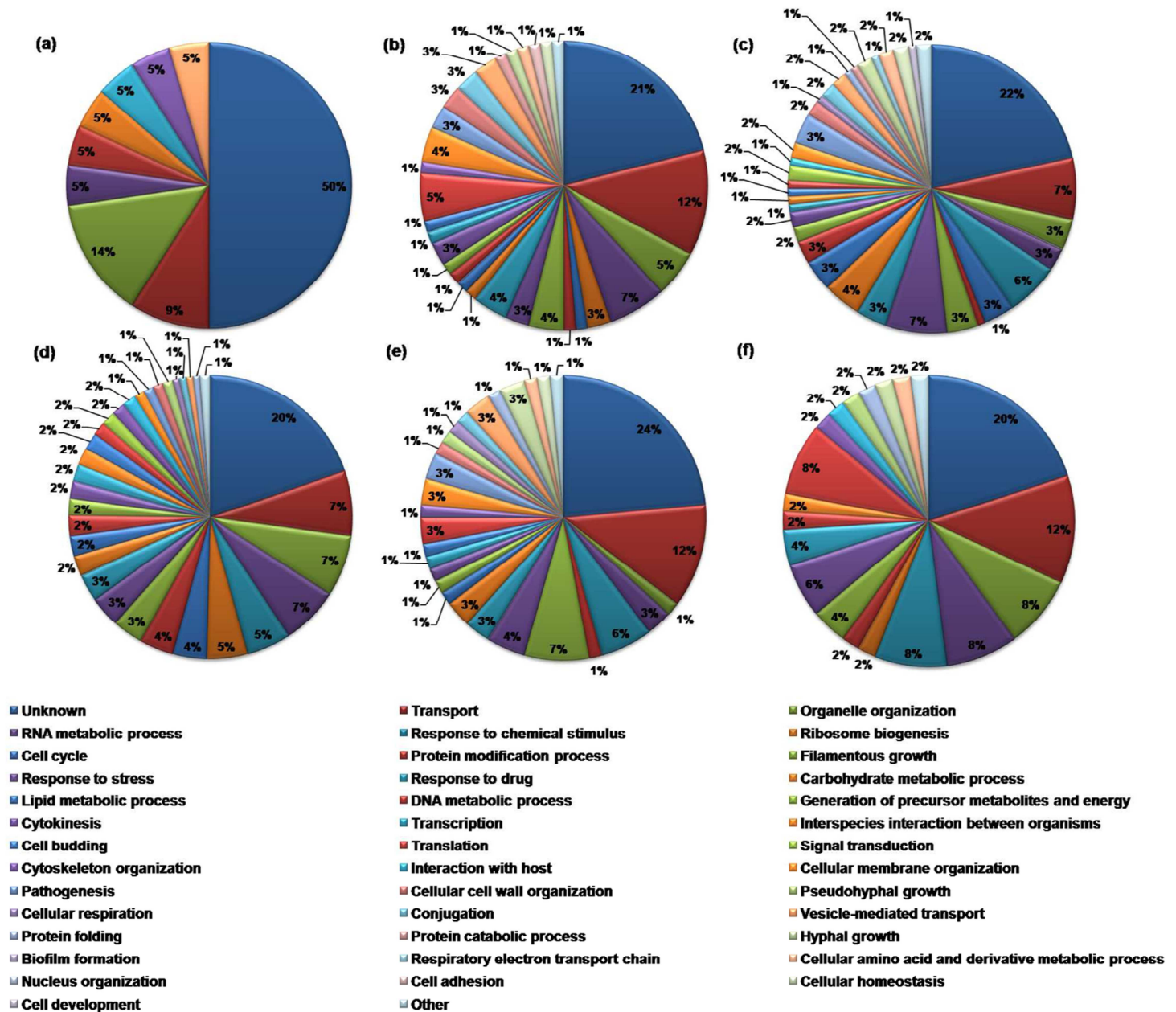


Figure 6. Pie charts indicating the percentage of the frequency of the *Candida albicans* genes, with their corresponding biological processes according to the Gene Ontology Slim Mapper from the *Candida* Genome Database (CGD), that have been differentially expressed during incubation at 37°C in the presence of 500 μM arachidonic acid. Genes up-regulated after (a) 8 h, (b) 24 h, (c) 48 h. Genes down-regulated after (d) 8 h, (e) 24 h, (f) 48 h.

4.4.1.1.1. Oxidoreductases

In order to identify possible enzymes involved in AA metabolism leading to eicosanoid production, genes encoding enzymes with oxidoreductase activity were studied. According to literature, a fatty acid desaturase (*OLE2*) and a multicopper oxidase (*FET3*) are involved in PGE₂ production by *C. albicans* (Erb-Downward & Noverr, 2007). However, these specific genes were not differentially expressed in the current study.

However, we identified seven other up-regulated oxidoreductase encoding genes that were up-regulated from 24–48 h. Their functions included amino acid synthesis (*LEU2*; orf19.7080), β -oxidation (*FOX2*, orf19.1288), carbohydrate metabolic processes (*GND1*, orf19.5024; *IFE2*, orf19.5288), a formate dehydrogenase (orf19.1117), a putative peroxidase (*TSA1*, orf19.7398.1) and a putative sulfiredoxin (orf19.3537) (Supplementary table). *IFE2*, encoding an alcohol dehydrogenase that is involved in sugar metabolism has increased expression in planktonic cells in response to prostaglandins (Levitin & Whiteway, 2007). Although we evaluated the effect of AA on *C. albicans* biofilms, compared to the study by Levitin and Whiteway (2007) where the effect of PGE₂ on the transcriptional responses of planktonic *C. albicans* cells were evaluated, a similar up-regulation of *IFE2* was observed (Figure 7). In our study, this could possibly be due to the production of PGE₂ from AA.

4.4.1.1.2. Hydrolases

Up-regulated genes encoding enzymes with hydrolase activity, especially at 24 and 48 h (Figure 5), included *HST6* (orf19.7440), *LEM3* (orf19.3542), *SCW11* (orf19.3893), *SNQ2* (orf19.5759), orf19.1029 and orf19.7392 (Supplementary table). *LEM3*, *SCW11* and *SNQ2* are also up-regulated after 24 and 48 h (Figure 7). *HST6*, *LEM3* and *SNQ2* also have transporter activities. The transport of a broad range of substrates across biological membranes occurs through the superfamily of the ATP binding cassette (ABC) proteins, which are also responsible for multidrug resistance (MDR) in microbial pathogens and cancer cells (Kovalchuk & Driessen, 2010).

4.4.1.1.3. Transporters

Yeasts have two recognized families of MDR transporters, ABC-MDR and the major facilitator superfamily-MDR (MFS-MDR) transporters (Prasad et al., 2011). In the model yeast, *S. cerevisiae*, Snq2p, an ABC-MDR protein, has been well studied and characterized as a pleiotropic drug resistance transporter (PDR) (Anderson et al., 2003; Kovalchuk & Driessen, 2010). In addition, Gaur and co-workers (2005) used the *C. albicans* genome sequence to identify 28 putative ABC proteins compared to the 22 in *S. cerevisiae* (Rogers et al., 2001). These *C. albicans* transporters were classified into six different subfamilies according to their domain organization, i.e. PDR, MDR, multidrug resistance-associated protein (MRP), the adrenoleukodystrophy protein (ALDp), the elongation factor-3 (EF3) and the RNase L inhibitor (RLI) (Gaur et al., 2005). Interestingly, *S. cerevisiae* contains an equivalent of each of these *C. albicans* subfamilies, suggesting a close relationship. Although, the molecular mechanism of the substrate specificity of these transporters is poorly understood, it is known that these subfamilies, share substrate specificity, between different yeasts and humans even though they have different sequence identity and inverted topology (Kolaczowski et al., 1998; Rogers et al., 2001).

The transport genes, *SNQ2* and *HST6*, which were significantly up-regulated in this study, belong to the PDR and MDR family, respectively. Recently, Hlaváček and co-workers (2009) indicated a possible physiological role of Snq2p, also a PDR transporter, in *S. cerevisiae* as well as a role in quorum sensing (QS), by exporting a possible QS metabolite out of the cell. It is known that QS is important during *C. albicans* biofilm formation (Enjalbert & Whiteway, 2005; Martins et al., 2007). Taken that this increased production of ROS may also be the case with *C. albicans* in the presence of AA, the over-expression of *SNQ2* could also lead to ROS-resistance as with *S. cerevisiae*. However, Alvarez and Konopka (2007) indicated that the sugar *N*-acetylglucosamine induces hyphal formation in *C. albicans* and through proteomic analysis that *SNQ2* is only involved in the budding phase.

In addition, it was found that the over-expression of the Snq2p in *S. cerevisiae*, mediates resistance to reactive oxygen species (ROS) such as singlet oxygen-generating compounds (Kovalchuk & Driessen, 2010; Ververidis et al., 2001). Additionally, AA was found to increase the production of ROS, such as

hydrogen peroxide, by bovine heart mitochondria (Cocco et al., 1999). Taken that this increased production of ROS may also be the case with *C. albicans* in the presence of AA, the over-expression of *SNQ2* could also lead to ROS-resistance, as with *S. cerevisiae*. The ABC transporter, MRP4, is involved in cellular signalling such as the export of oxygenated lipids, i.e. eicosanoids, such as thromboxane B₂, PGE₁, PGE₂, and PGF_{2α}, leukotrienes B₄ and C₄ out of epithelial cells of human seminal vesicles, from inside-out membrane vesicles derived from insect cells, HEK293 cells, V79 fibroblasts as well as vesicles from human platelets and myelomonocytic U937 cells (Reid et al., 2003; Rius et al., 2005, 2007). The export of PGE₂ across the blood-brain barrier in mouse is also due to MRP4 (Akanuma et al., 2010). Interestingly, Torky and co-workers (2008) indicated that the AA pathway leading to the production of PGE₂ activated the MRP transport function in primary epithelial lung cells. However, this was not true for PGF_{2α}. Similarly, in rice roots it was found that jasmonic acid, necessary for plant defence and secondary metabolism, induced the expression of PDR transporters (Moons, 2008). Nonsteroidal anti-inflammatory drugs (NSAIDs), known to inhibit prostaglandin production, was also found to inhibit the export of the different prostaglandins through MRP inhibition in mammalian cells (Reid et al., 2003; Torky et al., 2008). The *C. albicans* PDR subfamily, to which *SNQ2* belongs, shows reverse topology to the MDR and MRP subfamilies (Gaur et al., 2005). However, this does not mean that their functions differ, because as was previously mentioned, transporters with different conformation may have the same substrate specificity (Kolaczowski et al., 1998; Rogers et al., 2001). This suggests the possible role of *SNQ2* in PGE₂ export out of *C. albicans* cells.

In addition to the transport of oxygenated lipids, ABC transporters were also found to be involved in transport of other lipids in *C. albicans*. The ABC transporters of *C. albicans*, Cdr1p, Cdr2p and Cdr3p (*Candida drug resistance*) were found to be involved in the translocation of phospholipids, maintaining an asymmetric distribution of phospholipids between plasma membrane layers (Dogra et al., 1999; Smriti et al., 2002). This is similar to human Mdr1p and Mdr3p (Ruetz & Gros, 1994; Van Helvoort et al., 1996).

The other ABC transporter significantly up-regulated, *HST6*, belongs to the *C. albicans* MDR subfamily, but has weak homology to mammalian MDR proteins (Gaur

et al., 2005), and is homologous to Ste6p from *S. cerevisiae*, and the mammalian drug transporter P-glycoproteins (Pgps) (Raymond et al., 1998). *STE6* is known to be responsible for the transport of a mating pheromone, a-factor, in *S. cerevisiae* (Kuchler et al., 1989). Initially, *Candida* species were classified as anamorphs, with no sexual reproductive cycle (Poulter et al., 1981), but before mating was observed, a single *MTL* (mating-type like) locus which is normally heterozygous, *MTLa* and *MTL α* , had already been identified in *C. albicans* (Bennet et al., 2003; Bennett & Johnson, 2005). For *C. albicans* to mate, it must first undergo *MTL* homozygosis to *a/a* or α/α , and this was successfully obtained through laboratory manipulation (Magee & Magee, 2000) and later *in vivo* (Hull et al., 2000). During mating, homozygous cells secrete a mating pheromone (a or α factor). A receptor, encoded by *STE2*, on the surface of the opposite mating type reacts to this stimulus, allowing growth towards the source of the pheromone, eventually leading to the cells fusing with each other (Bennett et al., 2003; Magee et al., 2002). To confirm mating in *C. albicans*, Magee and co-workers (2002) found that most of the genes responsible for mating in *S. cerevisiae* are also involved in *C. albicans* mating, including *HST6*, which acts as an a-pheromone transporter. Additionally, it is known that for *C. albicans* to be able to mate, a phenotypic switch from the stable white phase to the less stable opaque phase has to occur (Bennett & Johnson, 2005). Interestingly, Raymond and co-workers (1998) found in different *C. albicans* cell types (i.e. yeast, hyphae, white and opaque cells) that *HST6* is constitutively expressed at high levels. This would suggest that in addition to the a-pheromone transporting activity of *HST6*, it has another biological function as a transporter. In addition, Ste6p, together with Pgp and MRP, was found to transport a synthetic lipid and phosphatidylcholine analogue, edelfosine, via a common flippase mechanism (Ruetz et al., 1997). This may suggest the involvement of these ABC transporters, including Hst6p, in the transport of physiological lipids.

In our study a putative PDR subfamily ABC transporter gene, orf19.4531 (Gaur et al., 2005), is amongst the five genes that were significantly down-regulated over the entire incubation period in the presence of AA (Figure 9). The only gene in our study that was significantly up-regulated after 8, 24 and 48 h, is orf19.4706 (Figure 7). Bennet and Johnson (2006) found that this gene was one of the genes induced (4-fold induction) in response to a-pheromone in SpiderM medium.

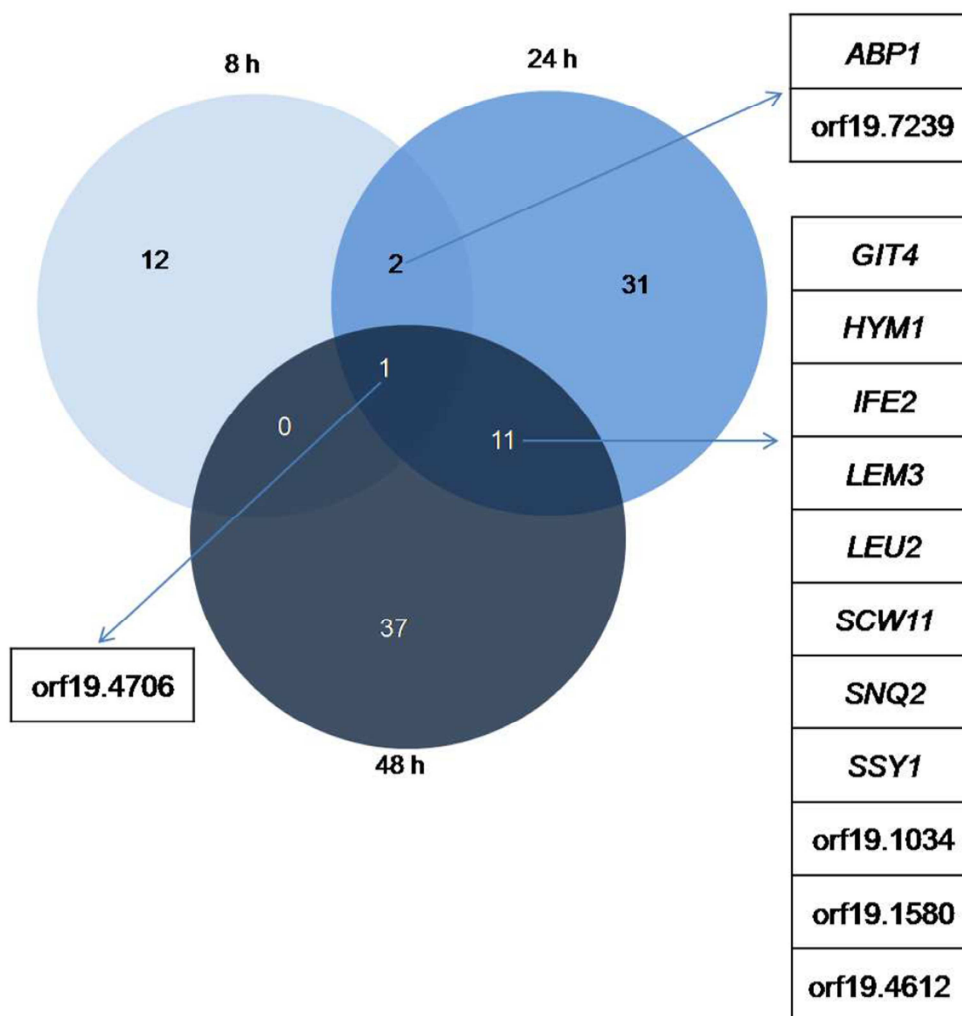


Figure 7. Venn Diagram illustrating the number of genes that were up-regulated in the presence of arachidonic acid during the 48 h incubation period compared to untreated *Candida albicans* biofilms. Also indicated are the common gene names or orf19 names of the genes that are up-regulated during the incubation period.

4.4.1.1.4. Response to oxidative stress

Free AA in the cells usually occurs due to the activity of phospholipases (Lambert, 1994). This AA can then be metabolized by various enzymes (Yilmaz, 2001), which may lead to the production of free radicals and peroxides resulting in oxidative damage to the cells (Katsuki & Okuda, 1995). It was found that these free radicals have a harmful effect by promoting membrane phospholipid degradation in the brain leading to more free fatty acids released as well as the inhibition of the reacylation of phospholipids by fatty acid hydroperoxides (Cummings et al., 2000;

Katsuki & Okuda, 1995). This oxidative change in the phospholipids affects membrane fluidity, cell signalling as well as protein structure.

A nuclear oxidoreductase gene, *TSA1*, was significantly up-regulated after 48 h (Supplementary table). This gene encodes a putative peroxidase that is responsible for resistance towards oxidative stress as well as cell wall biogenesis and locates to the nucleus of hyphal cells (Shin et al., 2005; Urban et al., 2005). This protein also regulates other genes in the nucleus under oxidative stress conditions, such as orf19.3537, encoding a putative sulfiredoxin (up-regulated after 24 h), and *GPD1* (orf19.1756), encoding a homologue of glycerol-3-phosphate dehydrogenase (up-regulated after 48 h) (Urban et al., 2005). In addition, the putative 6-phosphogluconate dehydrogenase, encoded by *GND1* (orf19.5024), was significantly up-regulated after 48 h in our study and also known to be involved in response to oxidative stress (Inglis et al., 2011).

The multidrug ABC transporter gene, *CDR1* (orf19.6000), as mentioned above, transport phospholipids in an in-to-out direction (Shukla et al., 2007; Smriti et al., 2002) and is also involved in response to oxidative stress (Inglis et al., 2011). In our study, we found that this gene was significantly down-regulated after 8 h (Supplementary table). This may be a reason for the reported increase in susceptibility of *C. albicans* biofilms to the azole drug, clotrimazole, when grown in the presence of AA (Ells et al., 2009; Tanabe et al., 2011). In addition, as mentioned above, the PDR ABC transporter, *SNQ2*, can also mediate resistance to ROS.

4.4.1.1.5. Carbohydrate metabolic process

Interestingly, most of the *C. albicans* genes involved in glycolysis were down-regulated in this study by the presence of AA (Figure 8). A glycolysis-specific regulator, the transcription factor *TYE7* (orf19.4941) (Askew et al., 2009), was significantly down-regulated after 8 h (Supplementary table). Tye7p is involved in the control of glycolysis and is a key regulator and activator of key enzymes during glycolysis. In our study the genes coding for these enzymes were down-regulated after 8 h i.e. *FBA1* (orf19.4618) (putative fructose-bisphosphate aldolase), *GLG1* (orf19.3325) (glycogenin glucosyltransferase), *PFK1* (orf19.3967)

(phosphofructokinase) and *TDH3* (orf19.6814) (NAD-linked glyceraldehyde-3-phosphate dehydrogenase) (Supplementary table). These genes are involved in glycolysis and gluconeogenesis. In contrast to these genes, a gene encoding a possible aldo/keto reductase, *GCY1* (orf19.6757), also regulated by Tye7p, was significantly up-regulated after 48 h. In contrast to our results, Levitin and Whiteway (2007) found that *GLG1* was up-regulated in planktonic *C. albicans* cells in the presence of PGE₂ after a 10 min treatment. The regulation observed for these genes in our study might suggest alternative carbon metabolic processes involved such as the glyoxylate cycle and β -oxidation of fatty acids.

FOX2 (orf19.1288), encoding a 3-hydroxyacyl-CoA epimerase, was significantly up-regulated after 48 h (Supplementary table). This gene is known to be required for the alternative carbon metabolism i.e. fatty acid beta-oxidation (Piekarska et al., 2006; Ramírez & Lorenz, 2009). Deva and co-workers (2000) demonstrated that *C. albicans* has the ability to utilise AA as sole carbon source to stimulate cell growth. However, *FOX2* mRNA could not be detected when grown in glucose which is in contrast to the high expression during growth in the presence of 18:1(n-9) (Ramírez & Lorenz, 2007). This alternative carbon metabolism is also believed to be the mechanism involved during infection by *C. albicans* due to the limited carbon in the infected sites.

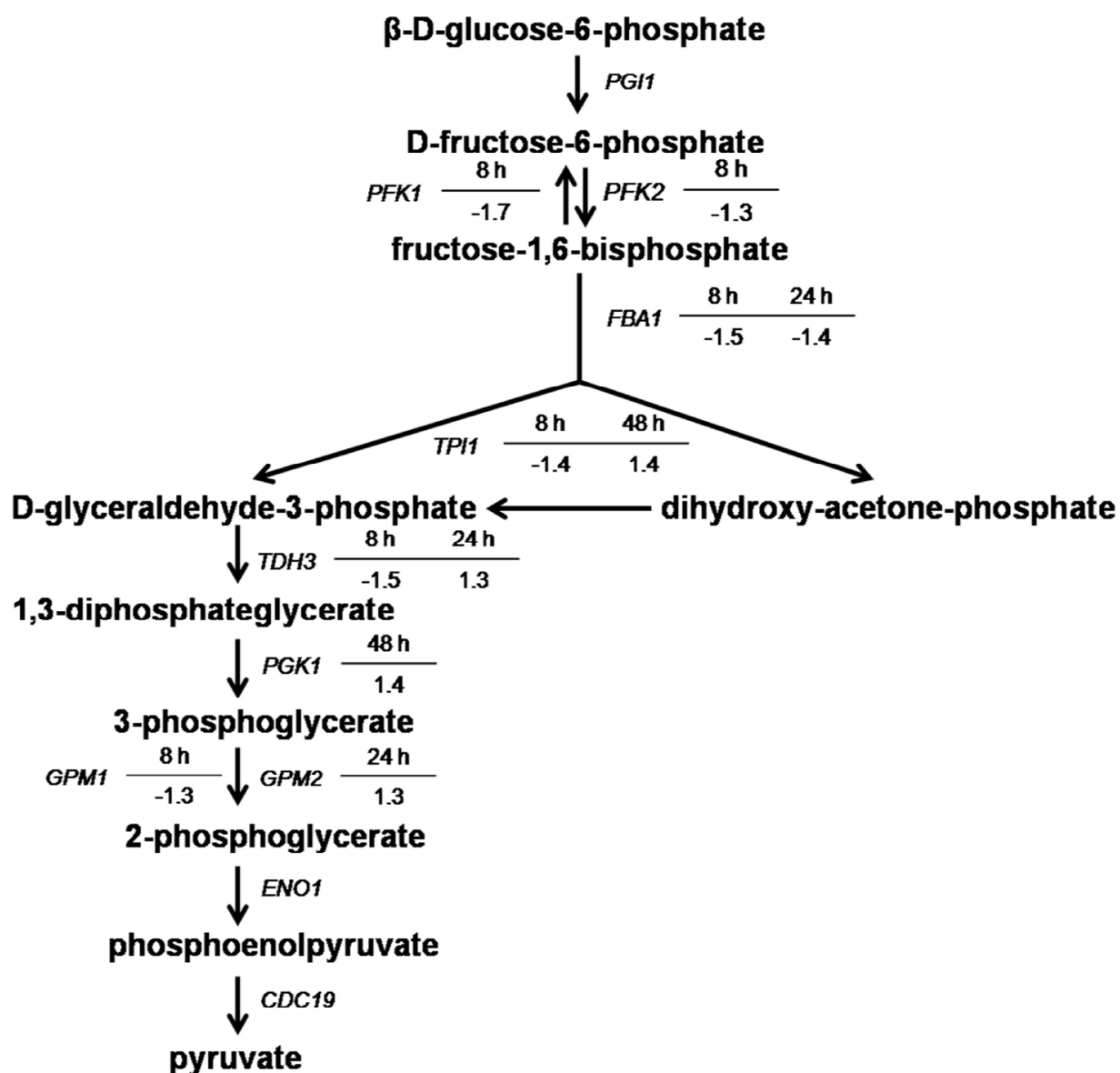


Figure 8. Schematic diagram of glycolysis, created by the *Candida* Genome Database (GCD), indicating the differentially expressed *Candida albicans* genes in the presence of arachidonic acid during a 48 h incubation period. Positive fold change indicates up-regulated genes and negative fold change down-regulated genes. Fold change values are relative to untreated sample at the same time point.

4.4.1.1.6. Other genes differentially expressed

The high-affinity iron permease gene, *FTR2* (orf19.7231) is amongst the five genes that were significantly down-regulated after 8, 24 and 48 h in the presence of

AA (Figure 9). Ramanan and Wang (2000) found that *C. albicans FTR2* is repressed when there is an iron deficiency and induced during sufficient iron supply. In addition, Cao and co-workers (2005) found a four-fold up-regulation of this gene when *C. albicans* biofilms were exposed to farnesol and suggested that this might contribute to an increase in azole susceptibility, due to the relationship between iron accessibility and azole resistance (Rogers & Barker, 2002). In fluconazole resistant isolates it was also found that *FTR2* was down-regulated compared to susceptible isolates where it was up-regulated. Similarly, Prasad and co-workers (2006) found that iron depletion led to more drug susceptible *C. albicans* cells caused by an increase in membrane fluidity and drug diffusion. These results might indicate that the presence of AA, down-regulated *FTR2* and might contribute to an increase in membrane fluidity in *C. albicans*.

In our study we found that *LDG3* (orf19.6486), a putative leucine, aspartic acid (D), glycine rich (LDG) family protein, was significantly down-regulated after 24 and 48 h, i.e. during the intermediate and mature phase of biofilm formation (Figure 9). Murillo and co-workers (2005) found that *LDG3* was one of the genes that have been significantly induced during the early stage of biofilm formation i.e. 30–390 min. However, we did not observe a significant effect after 8 h (480 min).

A nuclear protein with a high-mobility-group (HMG)-like acidic region, *MAK16* (orf19.5500), was significantly down-regulated after 8 h (Supplementary table). Levitin and Whiteway (2007) found that this gene was down-regulated in planktonic *C. albicans* cells in the presence of PGE₂ after a 10 min treatment. This again confirms the production of PGE₂ from as early as 8 h (Chapter 2).

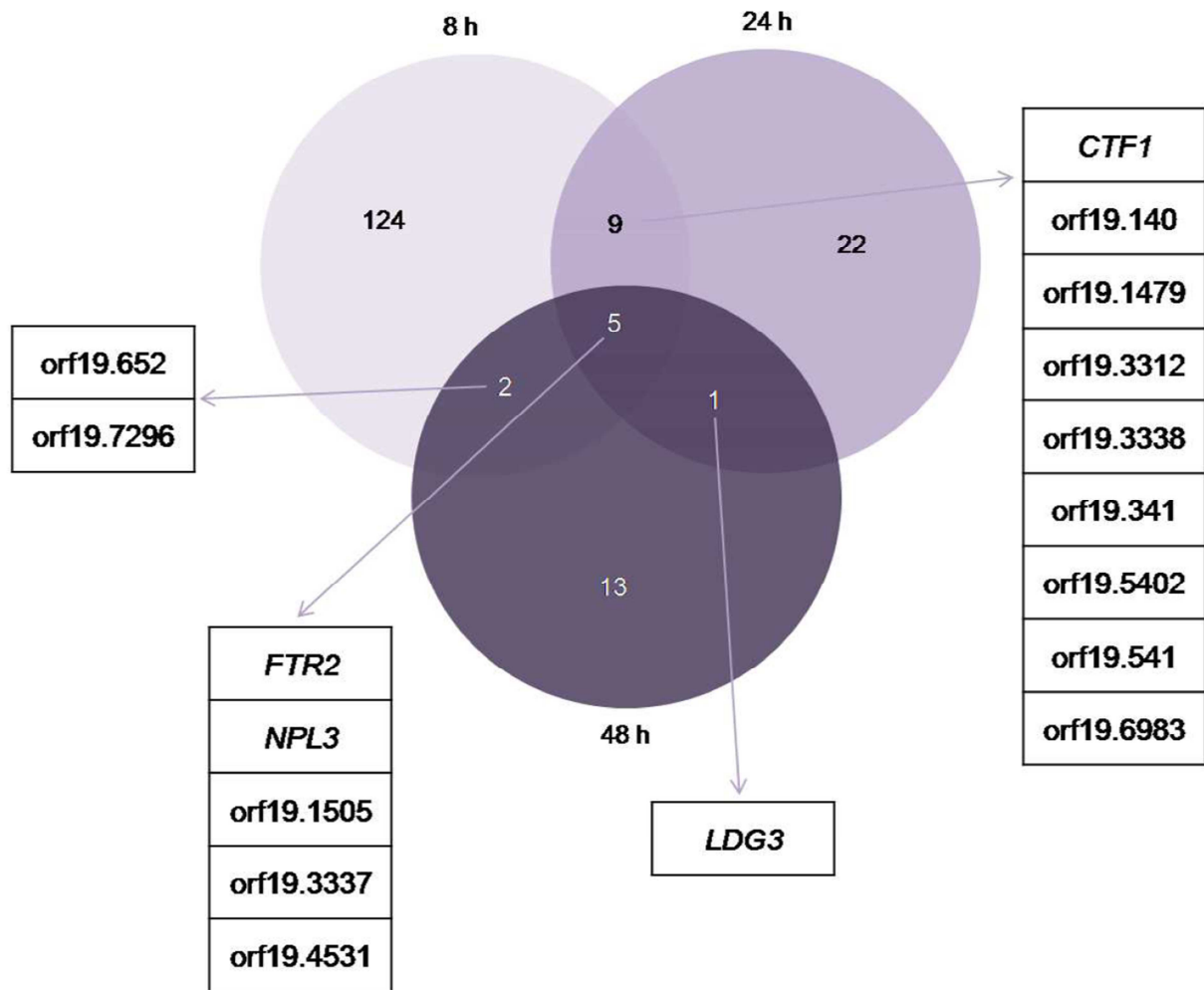


Figure 9. Venn Diagram illustrating the number of genes that are down-regulated in the presence of arachidonic acid during the 48 h incubation period compared to untreated *Candida albicans* biofilms. Also indicated are the common gene names or orf19 names of the genes that are down-regulated during the incubation period.

4.4.1.2. Cellular component

A cellular component is part of a cell or its extracellular environment which describe the locations at subcellular levels and macromolecules where a process occurs. Figure 10 indicates the cellular components of the differentially expressed genes in this study. It can be seen that most of the components are still unknown for both the up-regulated (Figure 10a-c) and down-regulated (Figure 10d-f) genes. As it was expected most of the known processes occur in the cytoplasm, as well as in the

membranes, nucleus and mitochondrion. Interestingly, in the membranes there is an increase in the processes from 8 up to 48 h. This can be due to the interaction and incorporation of AA into the cell membranes (Pérez et al., 2006).

4.4.1.2.1. Membranes

Fatty acids are the main components of lipids and play a key role as structural components of cellular membranes, affecting the physical state of the membranes and as storage lipids (Van Bogaert et al., 2011). In our study the presence of AA also affected membrane functions and membrane genes of *C. albicans*. It is known that AA is incorporated mainly into the phosphatidylinositol fraction of the phospholipids in the cell membranes (Ells, 2008; Tanaka et al., 2001). In our study we found that the inositol-1-phosphate synthase encoding gene, *INO1* (orf19.7585), which is responsible for the synthesis of inositol (Sreenivas et al., 2001), was significantly up-regulated after 8 h (Supplementary table). *ERG9* (orf19.3616), encodes a protein localized, amongst others, in the membranes and involved in ergosterol synthesis, was significantly up-regulated after 48 h (Supplementary table). This might possibly explain the increase in ergosterol in a *C. albicans* and *C. dubliniensis* strain previously found by us in the presence of AA (Ells et al., 2009). Membrane associated genes required for hyphal growth, such as *SSY1*, were also significantly up-regulated in the presence of AA (Brega et al., 2004) (Supplementary table).

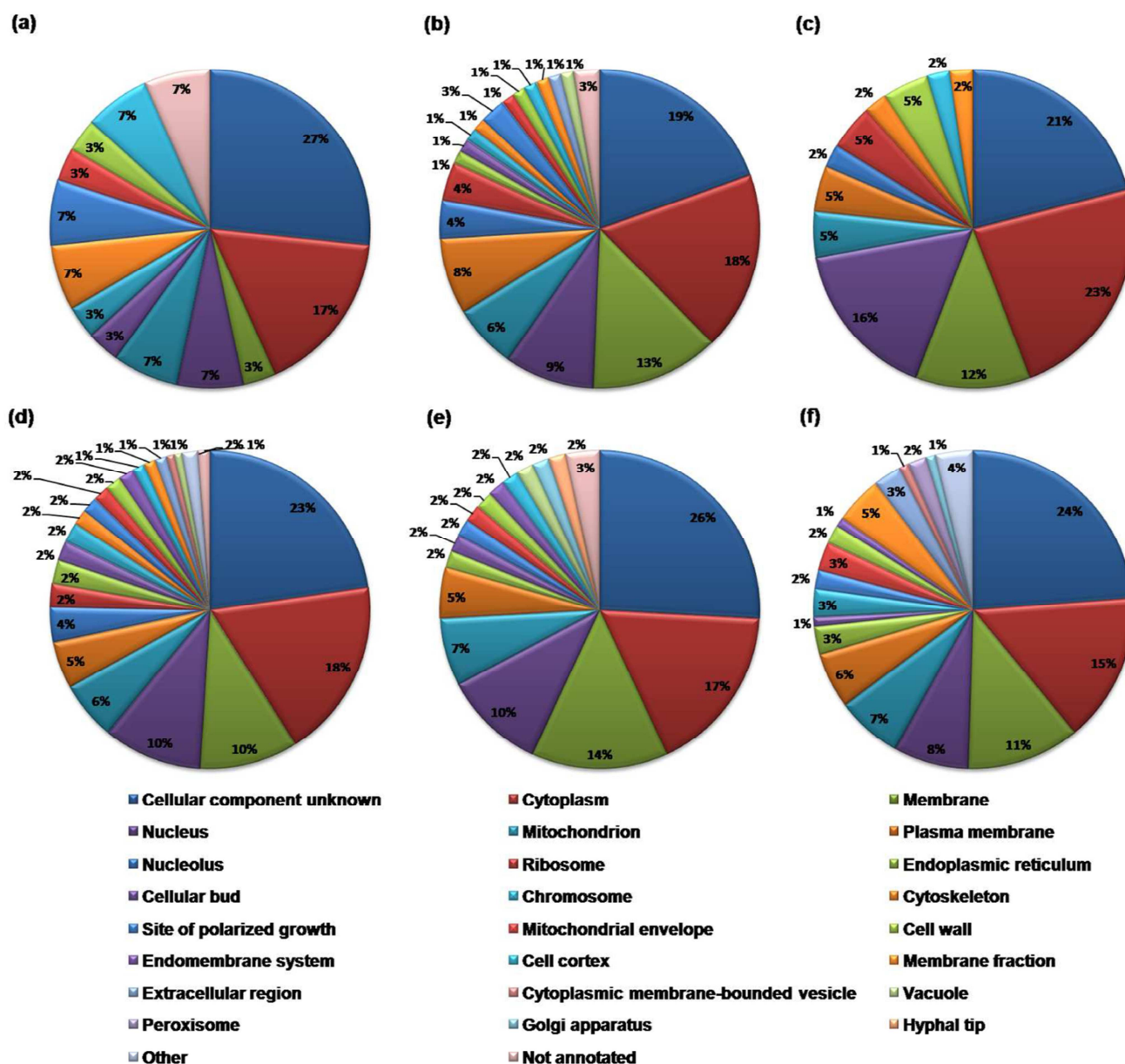


Figure 10. Pie charts indicating the cellular components of the differentially expressed *Candida albicans* genes according to the Gene Ontology Slim Mapper from the *Candida* Genome Database (CGD), which have been differentially expressed during incubation at 37°C in the presence of 500 µM arachidonic acid. Genes up-regulated after (a) 8 h, (b) 24 h, (c) 48 h. Genes down-regulated after (d) 8 h, (e) 24 h, (f) 48 h.

4.4.2. Two-Step Reverse transcriptase-qPCR analysis

A Two-Step RT-qPCR was done to confirm the expression of two genes, i.e. *SNQ2* and *HST6*. However, the regulation of *HST6* could not be confirmed. Table 3 indicates the comparison between the expression of *SNQ2* obtained through microarray and RT-qPCR analysis, where a similar pattern is observed especially after 48 h. During RT-qPCR, in addition to AA, we also evaluated the presence of SA on the expression of these two genes. This was done to evaluate if this effect was AA specific or if it is true for other fatty acids as well. It can be seen that although there is a slight decrease in the fold change in the presence of SA compared to the presence of AA, the trend is the same, suggesting that the up-regulation of *SNQ2* is not AA specific. In addition, since *C. albicans* cannot metabolize SA to PGE₂ (Chapter 3), this observed up-regulation of *SNQ2* is not due to the presence of PGE₂ but rather to the presence of 20-carbon polyunsaturated fatty acids.

The effect of AA and SA on *C. dubliniensis*, was also evaluated by the use of RT-qPCR. This was done to evaluate if the observed effect in *C. albicans* is a conserved characteristic. In these experiments, the same reference genes were used as for *C. albicans*, with primers designed specifically for these *C. dubliniensis* genes. However, through the use of the qBASE plus software, none of these reference genes were considered to be stably expressed by *C. dubliniensis* during these different treatments over the incubation period. For this reason, the expression of *C. dubliniensis* *SNQ2* (Cd36_64350) and *HST6* (Cd36_86480) could not be evaluated. This however needs further evaluation by the use of other reference genes.

Table 3: Comparison of the relative gene expression between microarray and Reverse transcriptase-qPCR analysis obtained for the selected *Candida albicans* genes in the presence of arachidonic acid (AA) and sciadonic acid (SA).

Gene	orf19 ID	Experiment	Microarray fold change [#]		RT-qPCR fold change*	
			AA	SA	AA	SA
SNQ2	orf19.5759	8 h	-		1.0	0.9
		24 h	1.5		0.8	0.6
		48 h	1.7		3.2	2.3

[#] Positive fold change indicates up-regulated genes and negative fold change down-regulated genes. Fold change values are relative to untreated sample at the same time point.

*Fold change values less than 1.0 indicates down-regulated genes and values of more than 1.0 up-regulated genes. Fold change values are relative to untreated sample at the same time point.

4.5. Conclusions

Microarray analysis is a useful tool that provides insight into the transcriptional state of a cell by measuring the mRNA levels of thousands of genes simultaneously. It enables the analysis of global changes in gene expression in the cell due to changes in the external and internal conditions. In this study, microarrays were used to identify *C. albicans* biofilm genes that are differentially expressed in the presence of AA. We found that a total of 4.1% of the 6346 *C. albicans* ORF's were differentially expressed over the incubation period. These genes had diverse functions not normally required for cell growth. The ABC transporter, SNQ2, was found to be regulated in this study and this is possibly due to the presence of 20-carbon fatty acids rather than PGE₂, since a similar trend was observed with both AA and SA. Although this was confirmed with RT-qPCR analysis, further research to elucidate the metabolic involvement of these genes needs to be conducted. It was also found that the presence of AA influenced the membrane functions, the carbohydrate metabolic processes and the response to oxidative stress in *C. albicans*.

It will also be interesting to evaluate the effect of these genes, or homologues of these genes, in other prostaglandin producing fungi as well as the use of other prostaglandin fatty acid precursors.

4.6. Acknowledgements

I would like to thank Mr. N. Olivier and Prof. D. Berger from the African Centre for Gene Technologies (ACGT), Microarray facility, at the University of Pretoria for the design of the microarray experiment, the use of their facilities as well as the statistical analysis of the data. I thank the post graduate students, as well as Dr. B. Visser, from Botany at the University of the Free State, Bloemfontein for assisting with the RT-qPCR experiments and the use of their facilities.

I thank the National Research Foundation, South Africa, for funding under the Thuthuka programme (Grant number TTK2007041000014), the Blue Skies programme (Grant number BS2008092300002) and the UFS Advanced Biomolecular Research Cluster programme.

4.7. References

- Akanuma S-i, Hosoya K-i, Ito SI, Tachikawa M, Terasaki T & Ohtsuki S. (2010). Involvement of multidrug resistance-associated protein 4 in efflux transport of prostaglandin E₂ across mouse blood-brain barrier and its inhibition by intravenous administration of cephalosporins. *J Pharmacol Exp Ther* 333: 912-919.
- Alem MAS & Douglas LJ. (2005). Prostaglandin production during growth of *Candida albicans* biofilms. *J Med Microbiol* 54: 1001-1005.
- Alvarez FJ & Konopka JB. (2007). Identification of an *N*-Acetylglucosamine transporter that mediates hyphal induction in *Candida albicans*. *Mol Biol Cell* 18: 965-975.
- Anderson JB, Sirjusingh C, Parsons AB, Boone C, Wickens C, Cowen LE & Kohn LM. (2003). Mode of selection and experimental evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. *Genetics* 163: 1287-1298.
- Askew C, Sellam A, Epp E, Hogues H, Mullick A, Nantel A & Whiteway M. (2009). Transcriptional regulation of carbohydrate metabolism in the human pathogen

Candida albicans. PLoS Pathog 5: e1000612. doi: 10.1371/journal.ppat.1000612.

Bennett RJ & Johnson AD. (2005). Mating in *Candida albicans* and the search for a sexual cycle. *Annu Rev Microbiol* 59: 233-255.

Bennett RJ & Johnson AD. (2006). The role of nutrient regulation and the Gpa2 protein in the mating pheromone response of *C. albicans*. *Mol Microbiol* 62: 100-119.

Bennett RJ, Uhl MA, Miller MG & Johnson AD. (2003). Identification and characterisation of a *Candida albicans* mating pheromone. *Mol Cell Biol* 23: 8189-8201.

Berger A & Jomard A. (2001). Fatty acid uninterrupted by a methylene as anti-inflammatory agents in superficial tissues of mammals. Patent no. US 6,280,755 B1.

Berger A, Monnard I, Baur M, Charbonnet C, Safonova I, Jomard A. (2002). Epidermal anti-inflammatory properties of 5,11,14 20:3: Effects on mouse ear edema, PGE₂ levels in cultured keratinocytes, and PPAR activation. *Lipids Health Dis* 1: 1-12.

Brega E, Zufferey R & Mamoun CB. (2004). *Candida albicans* Csy1p is a nutrient sensor important for activation of amino acid uptake and hyphal morphogenesis. *Eukaryot Cell* 3: 135-143.

Brown V, Sexton JA, Johnston M. (2006). A Glucose Sensor in *Candida albicans*. *Eukaryot Cell* 5: 1726-1737.

Cao Y-Y, Cao Y-B, Xu Z, Ying K, Li Y, Xie Y, Zhu Z-Y, Chen W-S & Jiang Y-Y. (2005). cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. *Antimicrob Agents Chemother* 49: 584-589.

- Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T & Ghannoum MA. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture and drug resistance. *J Bacteriol* 183: 5385-5394.
- Ciccoli R, Sahi S, Singh S, Prakash H, Zafiriou MP, Ishdorj G, Kock JLF & Nigam S. (2005). Oxygenation by COX-2 (cyclo-oxygenase-2) of 3-HETE (3-hydroxyeicosatetraenoic acid), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3- hydroxyeicosanoids. *J Biochem* 390: 737-747.
- Cocco T, Paola MD, Papa S & Lorusso M. (1999). Arachidonic acid interaction with the mitochondrial electron transport chain promotes reactive oxygen species generation. *Free Radical Bio Med* 27: 51-59.
- Crampton BG, Hein I, Berger DK. (2009). Salicylic acid confers resistance to a biotrophic rust pathogen, *Puccinia substriata*, in pearl millet (*Pennisetum glaucum*). *Molecular Plant Pathology* 10: 291-304.
- Cummings BS, Mchowat J & Schnellmann RG. (2000). Phospholipase A₂s in cell injury and death. *J Pharmacol Exp Ther* 294: 793-799.
- Deva R, Ciccoli R, Schewe T, Kock JLF & Nigam S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochim Biophys Acta* 1486: 299-311.
- Dogra S, Krishnamurthy S, Gupta V, Dixit BL, Gupta CM, Sanglard D & Prasad R. (1999). Asymmetric distribution of phosphatidylethanolamine in *C. albicans*: possible mediation by *CDR1*, a multidrug transporter belonging to ATP binding cassette (ABC) superfamily. *Yeast* 15: 111-121.
- Ells R. (2008). The effect of arachidonic acid on lipid metabolism and biofilm formation of two closely related *Candida* species. Magister Scientiae dissertation, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa. <http://etd.uovs.ac.za/ETD-db//theses/available/etd-10122009-095911/unrestricted/EllsR.pdf>

- Ells R, Kock JLF, van Wyk PWJ, Botes PJ & Pohl CH. (2009). Arachidonic acid increases antifungal susceptibility of *Candida albicans* and *Candida dubliniensis*. *J Antimicrob Chemother* 63: 124-128.
- Enjalbert B & Whiteway M. (2005). Release from quorum-sensing molecules triggers hyphal formation during *Candida albicans* resumption of growth. *Eukaryot Cell* 4: 1203-1210.
- Erb-Downward JR & Huffnagle GB. (2007). *Cryptococcus neoformans* produces authentic prostaglandin E₂ without a cyclooxygenase. *Eukaryot Cell* 6: 346-350.
- Erb-Downward JR, Noggle RM, Williamson PR & Huffnagle GB. (2008). The role of laccase in prostaglandin production by *Cryptococcus neoformans*. *Mol Microbiol* 68: 1428-1437.
- Erb-Downward JR & Noverr MC. (2007). Characterization of prostaglandin E₂ production by *Candida albicans*. *Infect Immun* 75: 3498-3505.
- Gaur M, Choudhury D & Prasad R. (2005). Complete inventory of ABC transporters in human pathogenic yeast, *Candida albicans*. *J Mol Microbiol Biotechnol* 9: 3-15.
- German JB, Gershwin ME & Berger A. (1995). Non-methylene interrupted fatty acids as immunomodulators. Patent no. 5,456,912.
- Hellemans J, Mortier G, De Paepe A, Speleman F & Vandesompele J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 8: R19 (doi: 10.1186/gb-2007-8-2-r19).
- Hlaváček O, Kučerová H, Harant K, Palková Z & Váchová L. (2009). Putative role for ABC multidrug exporters in yeast quorum sensing. *FEBS Letters* 583: 1107-1113.
- Hull CM, Raisner RM & Johnson AD. (2000). Evidence for mating of the “asexual” yeast *Candida albicans* in a mammalian host. *Science* 289: 307-310.

- Inglis DO, Arnaud MB, Binkley J, Shah P, Skrzypek MS, Wymore F, Binkley G, Miyasato SR, Simison M & Sherlock G. (2011). The *Candida* genome database incorporates multiple *Candida* species: multispecies search and analysis tools with curated gene and protein information for *Candida albicans* and *Candida glabrata*. *Nucleic Acids Res* doi: 10.1093/nar/gkr945.
- Jones T, Federspiel NA, Chibana H, Dungan J, Kalman S, Magee BB, Newport G, Thorstenson YR, Agabian N, Magee PT, Davis RW & Scherer S. (2004). The diploid genome sequence of *Candida albicans*. *Proc Natl Acad Sci* 101: 7329-7334.
- Kalo-Klein A & Witkin SS. (1990). Prostaglandin E₂ enhances and gamma interferon inhibits germ tube formation in *Candida albicans*. *Infect Immun* 58: 260-262.
- Katsuki H & Okuda S. (1995). Arachidonic acid as a neurotoxic and neurotrophic substance. *Prog Neurobiol* 46: 607-636.
- Kolaczkowski M, Kolaczowska A, Luczynski J, Witek S & Goffeau A. (1998). *In vivo* characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. *Microb Drug Resist* 4: 143-158.
- Kohrer K & Domdey H. (1991). Preparation of high molecular weight RNA. *Methods Enzymol* 194: 398-405.
- Kovalchuk A & Driessen AJM. (2010). Phylogenetic analysis of fungal ABC transporters. *BMC Genomics* 11: 177-197.
- Kuchler K, Sterne RE & Thorner J. (1989). *Saccharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. *EMBO J* 8: 3973-3984.
- Lambert IA. (1994). Eicosanoids and cell volume regulation. *Cellular and Molecular Physiology of Cell Volume Regulation*. CRC Press, London, Tokyo. pp 279-298.

- Levitin A & Whiteway M. (2007). The effect of prostaglandin E₂ on transcriptional responses of *Candida albicans*. *Microbiol Res* 162: 201-210.
- Magee BB, Legrand M, Alarco A-M, Raymond M & Magee PT. (2002). Many of the genes required for mating in *Saccharomyces cerevisiae* are also required for mating in *Candida albicans*. *Mol Microbiol* 46: 1345-1351.
- Magee BB & Magee PT. (2000). Induction of mating in *Candida albicans* by construction of *MTLa* and *MTL α* strains. *Science* 289: 310-313.
- Martins M, Henriques M, Azeredo J, Rocha SM, Coimbra MA & Oliveira R. (2007). Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot Cell* 6: 2429-2436.
- Moons A. (2008). Transcriptional profiling of the PDR gene family in rice roots in response to plant growth regulators, redox perturbations and weak organic acid stresses. *Plants* 229: 53-71.
- Moran G, Stokes C, Sascha T, Hube B, Coleman DC & Sullivan D. (2004). Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*. *Microbiology* 150: 3363-3382.
- Murillo LA, Newport G, Lan C-Y, Habelitz S, Dungan J & Agabian NM. (2005). Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot Cell* 4: 1562-1573.
- Naidoo S, Denby KJ & Berger, DK. (2005). Microarray experiments: considerations for experimental design. *S Afr J Sci* 101: 347-354.
- Nelson B, Kurischko C, Horecka J, Mody M, Nair P, Pratt L, Zougman A, McBroom LDB, Hughes TR, Boone C & Luca FC. (2003). RAM: a conserved signaling network that regulates Ace2p transcriptional activity and polarized morphogenesis. *Mol Biol Cell* 14: 3782-3803.

- Noverr MC & Huffnagle GB. (2004). Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect Immun* 72: 6206-6210.
- Noverr MC, Phare SM, Toews GB, Coffey MJ & Huffnagle GB. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* 69: 2957-2963.
- Noverr MC, Toews GB & Huffnagle GB. (2002). Production of prostaglandins and leukotrienes by pathogenic fungi. *Infect Immun* 70: 400-402.
- Pappas PG, Rex JH, Lee J, Hamill RJ, Larsen RA, Powderly W, Kauffman CA, Hyslop N, Mangino JE, Chapman S, Horowitz HW, Edwards JE & Dismukes WE. (2003). A prospective observational study of candidemia: Epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin Infect Dis* 37: 634-643.
- Pérez R, Matabosch X, Llebaria A, Balboa MA & Balsinde J. (2006). Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J Lipid Res* 47: 484-491.
- Piekarska K, Mol E, van den Berg M, Hardy G, van den Burg J, van Roermund C, MacCallum D, Odds F & Distel B. (2006). Peroxisomal fatty acid β -oxidation is not essential for virulence of *Candida albicans*. *Eukaryot Cell* 5: 1847-1856.
- Pfaller MA & Diekema DJ. (2010). Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol* 36: 1-53.
- Poulter R, Jeffery K, Hubbard MJ, Shepherd MG & Sullivan PA. (1981). Parasexual genetic analysis of *Candida albicans* by spheroplast fusion. *J Bacteriology* 146: 833-840.
- Prasad R, Sharma M & Rawal MK. (2011). Functionally relevant residues of Cdr1p: a multidrug ABC transporter of human pathogenic *Candida albicans*. *J Amino Acids* doi: 10.4061/2011/531412.
- Prasad T, Chandra A, Mukhopadhyay CK & Prasad R. (2006). Unexpected link between iron and drug resistance of *Candida* spp.: Iron depletion enhances

- membrane fluidity and drug diffusion, leading to drug-susceptible cells. *Antimicrob Agent Chemother* 50: 3597-3606.
- Ramage G, Vande Walle K, Wickes BL & Lopez-Ribot JL. (2001). Standardised method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob Agents Chemother* 45: 2475-2479.
- Ramanan N & Wang Y. (2000). A high-affinity iron permease essential for *Candida albicans* virulence. *Science* 288: 1062-1064.
- Ramírez MA & Lorenz MC. (2007). Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot Cell* 6: 280-290.
- Ramírez MA & Lorenz MC. (2009). The transcription factor homolog *CTF1* regulates β -oxidation in *Candida albicans*. *Eukaryot Cell* 8: 1604-1614.
- Raymond M, Dignard D, Alarco A-M, Mainville N, Magee BB & Thomas DY. (1998). A Ste6p/P-glycoprotein homologue from the asexual yeast *Candida albicans* transports the a-factor mating pheromone in *Saccharomyces cerevisiae*. *Mol Microbiol* 27: 587-598.
- Reid G, Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J & Borst P. (2003). The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proc Natl Acad Sci* 100: 9244-9249.
- Rius M, Hummel-Eisenbeiss J & Keppler D. (2007). ATP-dependent transport of leukotrienes B₄ and C₄ by the multidrug resistance protein ABCC4 (MRP4). *J Pharmacol Exp Ther* 324: 86-94.
- Rius M, Thon WF, Keppler D & Nies AT. (2005). Prostanoid transport by multidrug resistance protein 4 (MRP4/ABCC4) localized in tissues of the human urogenital tract. *J Urology* 174: 2409-2414.

- Rogers B, Decottignies A, Kolaczowski M, Carvajal E, Balzi E & Goffeau A. (2001). The pleiotropic drug ABC transporters from *Saccharomyces cerevisiae*. *J Mol Microbiol Biotechnol* 3: 207-214.
- Rogers PD & Barker KS. (2002). Evaluation of differential gene expression in fluconazole-susceptible and -resistant isolates of *Candida albicans* by cDNA microarray analysis. *Antimicrob Agent Chemother* 46: 3412-3417.
- Rozen S & Skaletsky HJ. (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds). *Bioinformatics methods and protocols: Methods in molecular biology*. Humana Press, Totowa, NJ, pp 365-386.
- Ruetz S, Brault M, Dalton W & Gros P. (1997). Functional interactions between synthetic alkyl phospholipids and the ABC transporters P-glycoprotein, Ste-6, MRP, and Pgh-1. *Biochemistry* 36: 8180-8188.
- Ruetz S & Gros P. (1994). Phosphatidylcholine translocase: a physiological role for the *mdr2* gene. *Cell* 77: 1071-1081.
- Shin DH, Jung S, Park SJ, Kim YJ, Ahn JM, Kim W & Choi W. (2005). Characterization of thiol-specific antioxidant 1 (*TSA1*) of *Candida albicans*. *Yeast* 22: 907-918.
- Shukla S, Rai V, Saini P, Banerjee D, Menon AK & Prasad R. (2007). *Candida* drug resistance protein 1, a major multidrug ATP binding cassette transporter of *Candida albicans*, translocates fluorescent phospholipids in a reconstituted system. *Biochemistry* 46: 12081-12090.
- Smriti, Krishnamurthy S, Dixit BL, Gupta CM, Milewski S & Prasad R. (2002). ABC transporters Cdr1p, Cdr2p and Cdr3p of a human pathogen *Candida albicans* are general phospholipid translocators. *Yeast* 19: 303-318.
- Smyth GK. (2005). Limma: linear models for microarray data. In *Bioinformatics and Computational Biology Solutions using R and Bioconductor*. Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W (eds.), Springer, New York, pages 397-420.

- Song Y, Cheon SA, Lee KE, Lee S-Y, Lee B-Y, Oh D-B, Kang HA & Kim J-Y. (2008). Role of the RAM network in cell polarity and hyphal morphogenesis in *Candida albicans*. *Mol Biol Cell* 19: 5456-5477.
- Sreenivas A, Villa-Garcia MJ, Henry SA & Carman GM. (2001). Phosphorylation of the yeast phospholipid synthesis regulatory protein Opi1p by protein kinase C. *J Biol Chem* 276: 29915-29923.
- Sullivan DJ, Moran GP, Pinjon E, Al-Mosaid A, Stokes C, Vaughan C & Coleman DC. (2004). Comparison of the epidemiology, drug resistance mechanisms and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res* 4: 369-376.
- Tanabe K, Lamping E, Nagi M, Okawada A, Holmes AR, Miyazaki Y, Cannon RD, Monk BC & Niimi M. (2011). Chimeras of *Candida albicans* Cdr1p and Cdr2p reveal features of pleiotropic drug resistance transporter structure and function. *Mol Microbiol* 82: 416-433.
- Tanaka T, Morishige J, Takimoto T, Takai Y & Satouchi K. (2001). Metabolic characterization of sciadonic acid as an effective substitute for arachidonate of phosphatidylinositol. *Eur J Biochem* 268: 4928-4939.
- Torky A, Raemisch A, Glahn F & Foth H. (2008). Arachidonic acid pathway activates multidrug resistance related protein in cultured human lung cells. *Arch Toxicol* 82: 323-332.
- Tsitsigiannis DI, Bok JW, Andes D, Nielsen KF, Frisvad JC & Keller NP. (2005). *Aspergillus* cyclooxygenase-like enzymes are associated with prostaglandin production and virulence. *Infect Immun* 73: 4548-4559.
- Urban C, Xiong X, Sohn K, Schröppel K, Brunner H & Rupp S. (2005). The moonlighting protein Tsa1p is implicated in oxidative stress response and in cell wall biogenesis in *Candida albicans*. *Mol Microbiol* 57: 1318-1341.
- Van Bogaert INA, Groeneboer S, Saerens K & Soetaert W. (2011). The role of cytochrome P450 monooxygenases in microbial fatty acid metabolism. *FEBS J* 278: 206-221.

- Van Helvoort A, Smith AJ, Sprong H, Fritzche I, Schinkel AH, Borst P & Van Meer G. (1996). MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* 87: 507-517.
- Ververidis P, Davrazou F, Diallinas G, Georgakopoulos D, Kanellis AK & Panopoulos N. (2001). A novel putative reductase (Cpd1p) and the multidrug exporter Snq2p are involved in resistance to cercosporin and other singlet oxygen-generating photosensitizers in *Saccharomyces cerevisiae*. *Curr Genet* 39: 127-136.
- Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP & Edmond MB. (2004). Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* 39: 309-317.
- Yeater KM, Chandra J, Cheng G, Mukherjee PK, Zhao X, Rodriguez-Zas SL, Kwast KE, Ghannoum MA & Hoyer LL. (2007). Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* 153: 2373-2385.
- Yilmaz E. (2001). Biotechnological production of prostaglandin. *Biotech Adv* 19: 387-397.

4.8. Supplementary table

Name	ID	<i>Candida</i> genome database description*	Fold change [#]		
			8 h	24 h	48 h
<i>NPL3</i>	orf19.7238	Putative RNA-binding protein; required for normal biofilm growth; nuclear export is facilitated by Hmt1p; transcription is up-regulated in an RHE model of oral candidiasis	-1.8	-2.7	-1.6
<i>FTR2</i>	orf19.7231	High-affinity iron permease; probably interacts with ferrous oxidase; regulated by iron level, ciclopirox olamine, amphotericin B, caspofungin; complements <i>S. cerevisiae</i> ftr1 iron transport defect; Hap43p-repressed	-1.6	-2.7	-1.5
	orf19.7296	Putative cation conductance protein; similar to stomatin mechanoreception protein; plasma-membrane localized; induced by Rgt1p	-2.8	-1.3	-1.6
	orf19.1505	Uncharacterized	-2.1	-2.1	-1.5
	orf19.3337	Late-stage biofilm-induced gene	-1.7	-1.8	-1.5
	orf19.4531	Putative PDR-subfamily ABC transporter	-1.6	-1.5	-1.7
<i>CTF1</i>	orf19.1499	Putative zinc-finger transcription factor, similar to <i>A. nidulans</i> FarA and FarB; activates genes required for fatty acid degradation; induced by oleate; null mutant displays carbon source utilization defects and slightly reduced virulence	-1.6	-1.8	-1.4
	orf19.6983	Hap43p-repressed gene; repressed by nitric oxide	-1.6	-1.7	-1.3
	orf19.1479	Uncharacterized	-1.5	-1.7	-1.4
	orf19.3312	<i>S. cerevisiae</i> ortholog Aim9p localizes to mitochondrion	-1.6	-1.7	-1.3
	orf19.729	Protein similar to <i>S. cerevisiae</i> She3p, an adaptor protein required for specific mRNA transport; transposon mutation affects filamentous growth	-1.8	-1.3	-1.4
	orf19.5045	Transcriptionally activated by Mnl1p under weak acid stress	-1.5	-1.4	-1.4
<i>AXL2</i>	orf19.5292	Protein not essential for viability; similar to <i>S. cerevisiae</i> Axl2p, which is a plasma membrane protein involved in determination of budding pattern; O-glycosylated by Pmt4p	-1.7	-1.3	-1.3
<i>HSL1</i>	orf19.4308	Probable protein kinase involved in determination of morphology during the cell cycle of both yeast-form and hyphal cells via regulation of Swe1p and Cdc28p; required for full virulence and kidney colonization in mouse systemic infection	-1.5	-1.3	-1.4
	orf19.7460	Putative 2'-O-methyltransferase with a predicted role in tRNA modification; transcription is activated in the presence of elevated CO ₂	-1.3	-1.3	-1.6
	orf19.2302	Uncharacterized	-1.3	-1.5	-1.4
<i>CCC1</i>	orf19.6948	Putative manganese transporter, required for normal filamentous growth; mRNA binds to She3p and is localized to hyphal tips; repressed by nitric oxide and alkaline pH; shows colony morphology-related regulation by Ssn6p; Hap43p-repressed	-1.3	-1.5	-1.4
<i>NBP35</i>	orf19.747	Similar to nucleotide-binding proteins; increased transcription is observed upon benomyl treatment; transcription is induced upon filamentous growth	-1.3	-1.4	-1.5
	orf19.117	<i>S. cerevisiae</i> ortholog Sec9p has SNAP receptor activity, has role in vesicle fusion and localizes to SNARE complex, extrinsic to plasma membrane	-1.5	-1.2	-1.3
	orf19.190	Uncharacterized	-1.5	-1.3	-1.3
	orf19.4738	<i>S. cerevisiae</i> ortholog Slp1p has role in protein folding in endoplasmic reticulum	-1.5	-1.1	-1.3

<i>TUB1</i>	orf19.7308	Alpha-tubulin; gene has intron; complements cold-sensitivity of <i>S. cerevisiae</i> tub1 mutant; <i>C. albicans</i> has single alpha-tubulin gene, whereas <i>S. cerevisiae</i> has two (TUB1, TUB3); farnesol-up-regulated in biofilm; sumoylation target	-2.2		-1.4
<i>HAT1</i>	orf19.779	<i>S. cerevisiae</i> ortholog Hat1p has histone binding, histone acetyltransferase activity, has role in histone acetylation, chromatin silencing at telomere and localizes to histone acetyltransferase complex, cytoplasm	-2.2	-1.2	
<i>LPG20</i>	orf19.771	Aldo-keto reductase family protein; similar to aryl alcohol dehydrogenases; osmotic stress-induced, correlates with overexpression of MDR1 in fluconazole-resistant isolate; stationary phase enriched protein	-2.1	-1.3	
<i>LDG3</i>	orf19.6486	Putative LDG family protein; induced upon biofilm formation		-1.9	-1.5
	orf19.341	Putative spermidine export pump (MDR-type pump); fungal-specific (no human or murine homolog)	-1.7	-1.6	
	orf19.3338	Uncharacterized	-1.6	-1.7	
<i>ZCF39</i>	orf19.7583	Putative transcription factor with zinc cluster DNA-binding motif; not essential for viability; filament induced		-1.9	-1.4
	orf19.140	Predicted ORF from Assembly 19; removed from Assembly 20	-1.6	-1.7	
	orf19.541	Uncharacterized	-1.7	-1.5	
	orf19.5402	ORF Predicted by Annotation Working Group; overlaps orf19.5401	-1.6	-1.5	
<i>HMI1</i>	orf19.7661	ATP-dependent 3' - 5' helicase involved in maintenance of mitochondrial DNA; ortholog of <i>S. cerevisiae</i> Hmi1p	-1.8	-1.3	
	orf19.104	Predicted ORF in Assemblies 19, 20 and 21; transcription is induced in response to alpha pheromone in SpiderM medium		-1.3	-1.8
	orf19.652	<i>S. cerevisiae</i> ortholog Yen1p has crossover junction endodeoxyribonuclease activity, has role in DNA repair and localizes to nucleus, cytoplasm	-1.7		-1.5
	orf19.1900	Uncharacterized	-1.7	-1.3	
	orf19.2016	<i>S. cerevisiae</i> ortholog Erg28p has protein binding, bridging, has role in ergosterol biosynthetic process and localizes to endoplasmic reticulum membrane	-1.7	-1.3	
<i>CBP1</i>	orf19.7323	Corticosteroid binding protein; transcription induced at late log-phase or upon adherence to polystyrene; not induced by corticosterone; contains a possible NAD/FAD binding region; regulated by Nrg1p, Tup1p		-1.6	-1.4
<i>PEX12</i>	orf19.2009	<i>S. cerevisiae</i> ortholog Pex12p has ubiquitin-protein ligase activity, has role in protein import into peroxisome matrix and localizes to integral to peroxisomal membrane	-1.6	-1.4	
<i>RPL5</i>	orf19.6541	Ribosomal protein; genes encoding cytoplasmic ribosomal subunits, translation factors, tRNA synthetases are downregulated upon phagocytosis by murine macrophages; Hap43p-induced gene	-1.7	-1.3	
<i>DDR48</i>	orf19.4082	Immunogenic stress-associated protein; regulated by filamentous growth pathways; induced by benomyl, caspofungin, ketoconazole or in azole-resistant strain; Hog1p, farnesol, alkaline down-regulated; stationary phase enriched; biofilm-induced		-1.5	-1.5
<i>CMK1</i>	orf19.5911	Putative calcium/calmodulin-dependent protein kinase II; expression regulated upon white-opaque switching; biochemically purified Ca ²⁺ /CaM-dependent kinase is soluble, cytosolic, monomeric, and serine-autophosphorylated; Hap43p-repressed	-1.8	-1.2	
<i>SUI1</i>	orf19.1280	Putative translation initiation factor; flucytosine induced; genes encoding ribosomal subunits, translation factors, and tRNA synthetases are down-regulated upon phagocytosis by murine macrophage	-1.4	-1.5	
<i>FBA1</i>	orf19.4618	Putative fructose-bisphosphate aldolase, enzyme of glycolysis; antigenic in murine/human infection; regulated by yeast-hyphal switch; induced by Efg1p, Gcn4p, Hog1p, biofilm, planktonic growth, or fluconazole; phagocytosis-repressed	-1.5	-1.4	

	orf19.6462	<i>S. cerevisiae</i> ortholog Aim27p has role in protein folding in endoplasmic reticulum and localizes to ER membrane protein complex	-1.2	-1.7	
<i>CBK1</i>	orf19.4909	Ser/Thr kinase of cell wall integrity pathway; mutants show abnormal morphology and aggregation; Mob2p associated; required for wild-type hyphal growth and transcriptional regulation of cell-wall-associated genes	-1.4	-1.5	
	orf19.2308	Hap43p-repressed gene; biofilm- and planktonic growth-induced gene		-1.5	-1.4
<i>GAL102</i>	orf19.3674	Putative UDP-glucose-4-epimerase; mutation confers hypersensitivity to toxic ergosterol analogue; overlaps orf19.3673	-1.6		-1.3
	orf19.5885	Putative U3 snoRNP protein; flucytosine induced	-1.7	-1.2	
	orf19.5876	Predicted ORF in Assemblies 19, 20 and 21; greater mRNA abundance observed in a <i>cyr1</i> homozygous null mutant than in wild type; transcription is induced in response to alpha pheromone in SpiderM medium		-1.4	-1.5
<i>SSA2</i>	orf19.1065	HSP70 family chaperone; found in cell wall fractions; antigenic; role in import of beta-defensin peptides; ATPase domain binds histatin 5; at surface of hyphae, not yeast-form cells; farnesol-down-regulated in biofilm; caspofungin repressed		-1.3	-1.6
<i>GRP2</i>	orf19.4309	Methylglyoxal reductase; regulation associated with azole resistance; induced in core stress response or by oxidative stress (via Cap1p), fluphenazine, benomyl, or with long term fluconazole treatment; Hap43p-induced; antigenic in humans	-1.6	-1.3	
<i>CRH12</i>	orf19.3966	Putative cell wall protein, member of the CRH family; transcription is regulated by Nrg1p and Tup1p; alkaline up-regulated by Rim101p; repressed during cell wall regeneration; biofilm-induced gene	-1.6	-1.3	
	orf19.2755	<i>S. cerevisiae</i> ortholog Pre7p has role in proteasomal ubiquitin-dependent protein catabolic process, proteasomal ubiquitin-independent protein catabolic process and localizes to proteasome core complex, beta-subunit complex	-1.7	-1.2	
	orf19.7295	Uncharacterized	-1.5		-1.3
<i>ERB1</i>	orf19.1047	Protein with a predicted role in ribosomal large subunit biogenesis; mutation confers hypersensitivity to 5-fluorocytosine (5-FC), 5-fluorouracil (5-FU), and tubercidin (7-deazaadenosine); hyphal repressed; macrophage repressed		-1.3	-1.6
<i>EFH1</i>	orf19.5498	Transcriptional activator; homodimer; minor role in transcriptional regulation vs Efg1p; regulates filamentous growth, phenotypic switching; EFG1 and EFH1 genetically interact; expression interferes with mouse intestinal tract colonization	-1.5	-1.3	
	orf19.1189	Late-stage biofilm-induced gene; transcription regulated upon yeast-hyphal switch; transcriptionally activated by Mnl1p under weak acid stress; contains a 5' UTR intron	-1.5	-1.3	
<i>MAK16</i>	orf19.5500	Putative constituent of 66S pre-ribosomal particles; Hap43p-induced gene; decreased expression in response to prostaglandins	-1.6	-1.2	
<i>MAS1</i>	orf19.3026	Putative mitochondrial processing protease; Hap43p-repressed gene; transcription is regulated by Nrg1p; oxidative stress-induced via Cap1p		-1.2	-1.6
<i>OST1</i>	orf19.6988	Putative oligosaccharyltransferase	-1.5	-1.3	

	orf19.5306	Uncharacterized	-1.5	-1.2	
	orf19.5195	<i>S. cerevisiae</i> ortholog Ura6p has uridylylate kinase activity, adenylate kinase activity, has role in 'de novo' pyrimidine base biosynthetic process and localizes to nucleus, cytoplasm		-1.2	-1.6
	orf19.6970	Protein not essential for viability	-1.5	-1.2	
<i>PSP1</i>	orf19.671	Protein repressed during the mating process	-1.6	-1.2	
<i>TBF1</i>	orf19.801	Essential transcriptional activator that regulates ribosomal protein genes and the rDNA locus; acts with Cbf1p at a subset of promoters; recruits Fhl1p and Lfh1p to promoters; role is analogous to that of <i>S. cerevisiae</i> Rap1p	-1.5	-1.2	
<i>RTF1</i>	orf19.7047	Putative RNA polymerase II-associated Paf1 complex subunit; induced during the mating process	-1.5	-1.2	
	orf19.2017	<i>S. cerevisiae</i> ortholog Rpa49p has role in transcription elongation from RNA polymerase I promoter, regulation of cell size, transcription of nuclear large rRNA transcript from RNA polymerase I promoter	-1.5	-1.1	
	orf19.3481	Putative mitochondrial ATP-dependent RNA helicase of the DEAD-box family, transcription is activated in the presence of elevated CO ₂	-1.5	-1.2	
<i>CDR1</i>	orf19.6000	Multidrug transporter of ATP-binding cassette (ABC) superfamily; transports phospholipids in an in-to-out direction; transcription induced by beta-estradiol, progesterone, corticosteroid, or cholesterol; repressed in young biofilms	-2.3		
	orf19.3332	Uncharacterized		-2.0	
	orf19.1480	Putative succinate dehydrogenase, enzyme of citric acid cycle; downregulated by Efg1p; repressed by nitric oxide; Hap43p-repressed gene		-2.0	
	orf19.1935	Uncharacterized	-2.0		
<i>MUP1</i>	orf19.5280	Putative high affinity methionine permease; alkaline upregulated by Rim101p		-2.0	
	orf19.6898	Predicted ORF in Assemblies 19, 20 and 21; increased transcription is observed upon benomyl treatment or in an azole-resistant strain that overexpresses MDR1	-1.9		
	orf19.2737	Ortholog of <i>S. cerevisiae</i> Ydr109cp	-1.9	-1.3	1.3
<i>KIC1</i>	orf19.191	Member of the GCK-III subfamily of eukaryotic Ste20p kinases; in RAM cell wall integrity signalling network; role in cell separation, azole sensitivity; required for hyphal growth; constitutive expression is MTL, white-opaque independent	-1.9		
	orf19.6008	Predicted ORF in Assemblies 19, 20 and 21; transcription is repressed in response to alpha pheromone in SpiderM medium	-1.8		
	orf19.6286	Ortholog of <i>S. cerevisiae</i> Yer128wp	-1.8		
	orf19.1340	Putative aldose reductase; protein level decreases in stationary phase cultures	-1.8		
	orf19.3325	Putative glycogen synthesis initiator; regulated by Efg1p and Efh1p; Hog1p-downregulated; shows colony morphology-related gene regulation by Ssn6p; increased expression in response to prostaglandins; biofilm-induced	-1.8		
<i>DBP7</i>	orf19.6902	Putative ATP-dependent DEAD-box RNA helicase; Hap43p-induced gene; transcription is upregulated in both intermediate and mature biofilms	-1.8		

<i>RPL38</i>	orf19.2111.2	60S ribosomal protein subunit; genes encoding cytoplasmic ribosomal subunits, translation factors, tRNA synthetases are downregulated upon phagocytosis by murine macrophage	-1.7		
	orf19.4824	Planktonic growth-induced gene	-1.7		
	orf19.7297	Putative cystathionine gamma-synthase; decreased levels in stationary phase cultures; Hog1p-induced; Gcn4p-regulated	-1.7		
<i>RBF1</i>	orf19.5558	Transcription factor; glutamine-rich activation domain; binds RPG-box DNA sequences; predominantly nuclear; mutation causes accelerated induction of filamentous growth; antigenic during human oral infection; Sko1p-repressed		-1.7	
<i>CCP1</i>	orf19.238	Similar to cytochrome-c peroxidase N terminus; transcription is negatively regulated by Rim101p or alkaline pH; transcription induced by interaction with macrophage or low iron; oxygen-induced activity; Hap43p-repressed gene		-1.7	
<i>UTP8</i>	orf19.5436	Essential protein involved in tRNA export from the nucleus and ribosomal small subunit biogenesis; physically interacts with TAP-tagged Nop1p	-1.7		
<i>PFK1</i>	orf19.3967	Phosphofructokinase alpha subunit, Pfk1p-Pfk2p heteromultimer; activated by fructose 2,6-bisphosphate, AMP, inhibited by ATP; activity reduced on hyphal induction; phagocytosis-down-regulated; fluconazole-induced; biofilm-induced	-1.7		
	orf19.7290	<i>S. cerevisiae</i> ortholog Lsm1p has RNA cap binding, has role in deadenylation-dependent decapping of nuclear-transcribed mRNA and localizes to mRNA cap binding complex, cytoplasmic mRNA processing body	-1.7		
	orf19.1609	<i>S. cerevisiae</i> ortholog Kri1p has role in endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA and LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA) and localizes to nucleolus	-1.7		
<i>GUT1</i>	orf19.558	Putative glycerol kinase; downregulated upon adherence to polystyrene; greater mRNA abundance observed in a <i>cyr1</i> homozygous null mutant than in wild type		-1.7	
	orf19.4315	Uncharacterized	-1.7		
<i>YOX1</i>	orf19.7017	Putative homeodomain-containing transcriptional repressor; periodic mRNA expression, peak at cell-cycle G1/S phase	-1.7		
	orf19.692	Hap43p-repressed gene	-1.6		
	orf19.479.2	<i>S. cerevisiae</i> ortholog Sec22p has SNAP receptor activity and has role in vesicle fusion with Golgi apparatus, retrograde vesicle-mediated transport, Golgi to ER, ER to Golgi vesicle-mediated transport	-1.6		
	orf19.4653	Transcriptionally regulated by iron; expression greater in low iron; similar to GPI-linked cell-wall proteins	-1.6		
<i>FCY2</i>	orf19.333	Purine-cytosine permease of pyrimidine salvage; mutation associated with resistance to flucytosine in clinical isolates; transposon mutation affects filamentation; farnesol-up-regulated in biofilm; planktonic growth-induced	-1.6		
	orf19.5735.3	Uncharacterized	-1.6		
<i>FAB1</i>	orf19.1513	Phosphatidylinositol 3-phosphate 5-kinase; required for hyphal growth on solid media, and for wild-type vacuolar morphology and acidification; not required for wild-type virulence in mouse systemic infection or for adherence to HeLa cells	-1.6		
	orf19.1617	Protein similar to <i>S. cerevisiae</i> Ydr282cp; transposon mutation affects filamentous growth; Hap43p-repressed gene	-1.6		
	orf19.2781	Predicted ORF in Assemblies 19, 20 and 21; possibly an essential gene, disruptants not obtained by UAU1 method	-1.6		

<i>TPO3</i>	orf19.4737	Possible role in polyamine transport; MFS-MDR family; transcription induced by Sfu1p, regulated upon white-opaque switching; decreased expression in hyphae compared to yeast-form cells; regulated by Nrg1p; fungal-specific	-1.6		
	orf19.6601	Biofilm-induced gene	-1.6		
	orf19.849	<i>S. cerevisiae</i> ortholog Mnn4p has role in protein O-linked glycosylation, response to stress, protein N-linked glycosylation	-1.6		
<i>TYE7</i>	orf19.4941	bHLH transcription factor involved in control of glycolysis; required for biofilm formation; hyphally regulated by Cph1p, Cyr1p; flucytosine, Hog1p induced; amphotericin B, caspofungin repressed; induced in biofilm and planktonic growth	-1.6		
	orf19.5442	Uncharacterized	-1.6		
	orf19.7042	Increased transcription is observed upon benomyl treatment or in an azole-resistant strain that overexpresses MDR1; induced by nitric oxide	-1.6		
	orf19.6137	Uncharacterized	-1.6		
	orf19.1812	Predicted ORF from Assembly 19; merged with orf19.3071 in Assembly 20	-1.6		
<i>MIH1</i>	orf19.3071	Putative protein phosphatase of the PTP family (tyrosine-specific), similar to <i>S. cerevisiae</i> Mih1p; mRNA binds to She3p			
	orf19.7581	Uncharacterized	-1.6		
<i>COX5</i>	orf19.4759	Cytochrome oxidase subunit V; putative upstream CCAAT box regulatory element; macrophage/pseudohyphal-induced; repressed by nitric oxide; intron in 5'-UTR; Hap43p-dependent repression in low iron medium	-1.6		
<i>ASR3</i>	orf19.842	Gene regulated by cAMP and by osmotic stress; greater mRNA abundance observed in a <i>cyr1</i> or <i>ras1</i> homozygous null mutant than in wild type	-1.6		
	orf19.5300	Predicted ORF in Assemblies 19, 20 and 21; caspofungin induced	-1.6		
<i>RPL19A</i>	orf19.5904	Ribosomal protein L19; genes encoding cytoplasmic ribosomal subunits, translation factors, tRNA synthetases are down-regulated upon phagocytosis by murine macrophages; Hap43p-induced gene			-1.6
	orf19.2455	<i>S. cerevisiae</i> ortholog Dug2p has omega peptidase activity, has role in glutathione catabolic process and localizes to nucleus, cytoplasm	-1.6		
	orf19.2164	Transcription is a-specific, alpha-factor induced; merged with orf19.2219 in Assembly 20	-1.6		
<i>ORF298</i>	orf19.2219	Predicted ORF in retrotransposon Tca3 with similarity to Gag-Pol, which encodes nucleocapsid-like protein, reverse transcriptase, protease, and integrase; transcription is a-specific, alpha-factor induced; mRNA binds to She3p			
<i>TEN1</i>	orf19.3255	Protein involved in telomere maintenance; forms a complex with Stn1p; transcription is regulated upon yeast-hyphal switch	-1.6		
<i>SIK1</i>	orf19.7569	Putative U3 snoRNP protein; Hap43p-induced gene; physically interacts with TAP-tagged Nop1p	-1.6		
	orf19.7307	Putative oxidoreductase; similar to <i>S. cerevisiae</i> Pga3p; possible Kex2p substrate	-1.6		
	orf19.3706	Uncharacterized	-1.6		
<i>TPM2</i>	orf19.6414.3	Putative tropomyosin isoform 2; regulated by Gcn4p; repressed under amino acid starvation; macrophage-induced; protein levels decrease in stationary cells; Hap43p-induced gene	-1.6		
	orf19.230	Hap43p-induced gene	-1.6		

	orf19.6596	Putative esterase; possibly transcriptionally regulated by Tac1p; transcriptionally activated by Mnl1p under weak acid stress; protein present in exponential and stationary growth phase yeast cultures	-1.6		
<i>MYO1</i>	orf19.6294	Component of actomyosin ring at neck of newly-emerged bud	-1.6		
<i>CRH11</i>	orf19.2706	GPI-anchored cell wall transglycosylase, putative ortholog of <i>S. cerevisiae</i> Crh1p; predicted glycosyl hydrolase domain; similar to Csf4p and to antigenic <i>A. fumigatus</i> Asp9; predicted Kex2p substrate; caspofungin-induced	-1.6		
	orf19.6715	Uncharacterized		-1.5	
	orf19.7596	Hap43p-repressed gene	-1.5		
	orf19.4061	Predicted ORF in Assemblies 19, 20 and 21; clade-associated gene expression		-1.5	
<i>CTM1</i>	orf19.7612	Putative cytochrome c lysine methyltransferase; early-stage biofilm-induced gene; regulated by Gcn2p and Gcn4p; transcriptionally activated by Mnl1p under weak acid stress	-1.5		
<i>PRN4</i>	orf19.2461	Protein with similarity to pirins; increased transcription is observed upon benomyl treatment	-1.5		
	orf19.6457	Ortholog of <i>S. cerevisiae</i> Ybl086cp		-1.5	
<i>RPP2B</i>	orf19.5928	Conserved acidic ribosomal protein, likely to be involved in regulation of translation elongation; interacts with Rpp1Ap; one of four similar <i>C. albicans</i> ribosomal proteins (Rpp1Ap, Rpp1Bp, Rpp2Ap, Rpp2Bp); macrophage/pseudohyphal-induced			-1.5
<i>RPL8B</i>	orf19.6002	Predicted ribosomal protein; transcription is regulated upon yeast-hyphal switch; genes encoding cytoplasmic ribosomal subunits, translation factors, and tRNA synthetases are down-regulated upon phagocytosis by murine macrophage	-1.5		
<i>KGD1</i>	orf19.6165	Putative 2-oxoglutarate dehydrogenase; regulated by Efg1p under yeast-form but not hyphal growth conditions; transcription is up-regulated in an RHE model of oral candidiasis; stationary phase enriched protein; Hap43p-repressed gene	-1.5		
	orf19.2684	Uncharacterized	-1.5		
	orf19.3184	Uncharacterized		-1.5	
	orf19.1507	Putative negative regulator of exit from mitosis; Plc1p-regulated	-1.5		
	orf19.5276	Putative nuclear pore-associated protein; Hap43p-induced gene; induced upon low-level peroxide stress; possibly an essential gene, disruptants not obtained by UAU1 method	-1.5		
<i>MSI3</i>	orf19.2435	Antigenic HSP70 family protein; functional homolog of <i>S. cerevisiae</i> Msi3p; interacts by 2-hybrid with Cgr1p; transcriptionally regulated by iron; expression greater in high iron; farnesol down-regulated in biofilm; sumoylation target	-1.5		
<i>YHB1</i>	orf19.3707	Nitric oxide dioxygenase, acts in nitric oxide scavenging/detoxification; role in virulence in mouse; transcription activated by NO, macrophage interaction; hyphal down-regulated; mRNA binds to She3p; Hap43p-repressed	-1.5		
	orf19.5905	Hap43p-induced gene; induced upon biofilm formation	-1.5		
<i>REI1</i>	orf19.59	Putative cytoplasmic pre-60S factor; Hap43p-induced gene; decreased expression in response to prostaglandins	-1.5		

<i>HAL9</i>	orf19.3190	Protein with Zn(2)-Cys(6) binuclear cluster; gene in zinc cluster region of Chr. 5; transcriptionally activated by Mnl1p in weak acid; similar to <i>S. cerevisiae</i> Hal9p, which is a putative transcription factor involved in salt tolerance		-1.5	
	orf19.1360.1	Putative adhesin-like protein	-1.5		
	orf19.6484	Uncharacterized			-1.5
	orf19.7004	Predicted ORF from Assembly 19; removed from Assembly 20		-1.5	
	orf19.2376	Ortholog of <i>C. glabrata</i> Cagl0k08932gp			
	orf19.6737	Protein not essential for viability; greater mRNA abundance observed in a <i>cyr1</i> , <i>ras1</i> , or <i>efg1</i> homozygous null mutant than in wild type	-1.5		
	orf19.1804	<i>S. cerevisiae</i> ortholog Ydl119cp localizes to mitochondrion	-1.5		
<i>RPS13</i>	orf19.4193.1	Putative ribosomal protein of the small subunit	-1.5		
	orf19.5980	Hap43p-repressed gene; planktonic growth-induced gene	-1.5		
	orf19.5284	Uncharacterized	-1.5		
	orf19.3887	Ortholog of <i>C. glabrata</i> Cagl0j06666gp	-1.5		
	orf19.3724	Ortholog of <i>S. cerevisiae</i> Utp23p	-1.5		
<i>JEN2</i>	orf19.5307	Dicarboxylic acid transporter; regulated by glucose repression; induced by Rgt1p; transcription is up-regulated in both intermediate and mature biofilms; disruptants not obtained by UAU1 method	-1.5		
<i>KSR1</i>	orf19.6131	3-ketosphinganine reductase, catalyzes the second step in phytosphingosine synthesis	-1.5		
	orf19.1334	Predicted ORF in Assemblies 19, 20 and 21; transcription is repressed in response to alpha pheromone in SpiderM medium	-1.5		
<i>NGG1</i>	orf19.3023	<i>S. cerevisiae</i> ortholog Ngg1p has histone acetyltransferase activity, has role in histone acetylation and localizes to SAGA complex, Ada2/Gcn5/Ada3 transcription activator complex, SLIK (SAGA-like) complex			-1.5
<i>ARG5,6</i>	orf19.4788	Arginine biosynthetic enzyme activities; in <i>S. cerevisiae</i> , processed into distinct polypeptides with acetylglutamate kinase (Arg6p) activity and acetylglutamate-phosphate reductase (Arg5p) activity; Gcn4p regulated; alkaline down-regulated			-1.5
	orf19.639	Predicted ORF in Assemblies 19, 20 and 21; decreased transcription is observed upon fluphenazine treatment or in an azole-resistant strain that overexpresses CDR1 and CDR2	-1.5		
<i>GRP1</i>	orf19.4781	Protein similar to dihydroflavonol-4-reductases			-1.5
	orf19.1830	Hap43p-induced gene	-1.5		
<i>CSE4</i>	orf19.6163	Centromere-associated protein; essential; 4 Cse4p-containing nucleosomes, each containing 2 Cse4p molecules, bind to each centromere; similar to CENP-A (centromeric histone H3 variant) proteins; Cse4p and Mif2p colocalize at centromeres	-1.5		
	orf19.3965	Uncharacterized	-1.5		
	orf19.6315	Uncharacterized	-1.9	1.2	

<i>LIP9</i>	orf19.5172	Secreted lipase, member of a lipase gene family whose members are expressed differentially in response to carbon source and during infection; may have a role in nutrition and/or in creating an acidic microenvironment	-1.7	1.2	
	orf19.1522	Uncharacterized	-1.6		1.3
	orf19.3661	Putative deubiquitinating enzyme; transcriptionally activated by Mnl1p under weak acid stress	-1.6	1.3	
	orf19.6789	Hap43p-induced gene	-1.5	1.2	
	orf19.2730	Uncharacterized	-1.7		1.4
<i>LAB5</i>	orf19.2774	<i>S. cerevisiae</i> ortholog Lip5p has role in protein lipoylation and localizes to mitochondrion	-1.7		1.4
	orf19.7566	Late-stage biofilm-induced gene; transcription is up-regulated in clinical isolates from HIV ⁺ patients with oral candidiasis; alkaline up-regulated by Rim101p; fungal-specific (no human or murine homolog)	1.2		-1.5
<i>BBC1</i>	orf19.2791	Putative SH3-domain-containing protein	1.2		-1.5
	orf19.6219	Uncharacterized	-1.5		1.3
<i>TDH3</i>	orf19.6814	NAD-linked glyceraldehyde-3-phosphate dehydrogenase; enzyme of glycolysis; binds fibronectin and laminin; at surface of yeast and hyphae; antigenic during infection; farnesol-down-regulated; stationary phase-enriched; biofilm-induced	-1.5	1.3	
	orf19.7167	Putative adhesin-like protein	1.3	-1.5	
	orf19.1709	Predicted ORF in Assemblies 19, 20 and 21; alkaline downregulated; shows colony morphology-related gene regulation by Ssn6p	1.5		-1.5
	orf19.5313	Predicted ORF from Assembly 19; merged with orf19.1430 in Assembly 20	-1.2	1.5	
	orf19.1430	Uncharacterized			
<i>VPS22</i>	orf19.6296	Putative ESCRT-II complex protein with a role in multivesicular body (MVB) trafficking; Hap43p-repressed gene	-1.7	1.4	1.4
	orf19.3450.1	Uncharacterized		1.5	
<i>POX1</i>	orf19.5723	Predicted acyl-CoA oxidase; expression is regulated upon white-opaque switching; up-regulated upon phagocytosis			1.5
	orf19.3852	Uncharacterized		1.5	
<i>ELF1</i>	orf19.7332	Putative mRNA export protein; Walker A and B (ATP/GTP binding) motifs, ABC transporter consensus; required for wild-type morphology, growth; expressed in hyphal, pseudohyphal, and yeast form, up-regulated in biofilm; Hap43p-induced gene		1.5	
	orf19.2317	Predicted ORF in Assemblies 19, 20 and 21; transcription is induced in response to alpha pheromone in SpiderM medium			1.5
	orf19.3643	Hap43p-repressed gene			1.5
	orf19.2431	Dubious open reading frame, not conserved in <i>Candida</i> species; transcription is induced in response to alpha pheromone in SpiderM medium; mRNA binds She3p	1.5		
	orf19.4883	Predicted ORF in Assemblies 19, 20 and 21; constitutive expression independent of MTL or white-opaque status	1.5		
	orf19.6864	Uncharacterized			1.5
	orf19.5057	Uncharacterized		1.5	

	orf19.4307	<i>S. cerevisiae</i> ortholog Did2p has role in late endosome to vacuole transport via multivesicular body sorting pathway, protein targeting to vacuole and localizes to late endosome	1.5		
<i>DAD3</i>	orf19.3871	Subunit of the Dam1 (DASH) complex, which acts in chromosome segregation by coupling kinetochores to spindle microtubules			1.5
<i>PHO84</i>	orf19.655	High-affinity phosphate transporter; expression regulated upon white-opaque switching or biofilm formation; Hog1p, ciclopirox olamine or alkaline induced; caspofungin or stress repressed; upregulated in RHE model; biofilm-, Hap43p-induced			1.5
	orf19.5543	Ortholog of <i>C. glabrata</i> Cagl0m04653gp			1.5
<i>RPS21B</i>	orf19.3325.3	Protein similar to ribosomal protein S21; regulated by Nrg1p, Tup1p; shows colony morphology-related gene regulation by Ssn6p; transcription is positively regulated by Tbf1p, Hap43p			1.5
	orf19.3586	Uncharacterized	1.5		
<i>LAP41</i>	orf19.1628	Putative aminopeptidase yscl precursor; not essential for viability; protein present in exponential and stationary growth phase yeast cultures			1.5
	orf19.7588	<i>S. cerevisiae</i> ortholog Rrg7p localizes to mitochondrion			1.5
<i>PGA59</i>	orf19.2767	Adhesin-like cell wall protein; putative GPI-anchor; shows colony morphology-related gene regulation by Ssn6p; Hap43p-induced gene			1.5
	orf19.5932	Ortholog of <i>S. cerevisiae</i> Ylr241wp	1.5		
<i>DAL5</i>	orf19.309	Allantoate permease; nitrogen catabolite repressed, induced in absence of preferred N sources; nitrogen source regulation requires Gat1p; fungal-specific (no human/murine homolog); possibly essential gene (by UAU1 method); Hap43p-repressed			1.6
	orf19.6859	Uncharacterized			1.6
	orf19.7392	<i>S. cerevisiae</i> ortholog Ded1p has ATP-dependent RNA helicase activity, RNA strand annealing activity and has role in translational initiation			1.6
	orf19.5508	Uncharacterized			1.6
	orf19.625	Predicted ORF from Assembly 19; removed from Assembly 20	1.6		
<i>GCR3</i>	orf19.387	Functional homolog of <i>S. cerevisiae</i> Gcr3p, which acts in regulation of glycolytic genes; no intron predicted, in contrast to intron in <i>S. cerevisiae</i> GCR3 gene			1.6
<i>FOX2</i>	orf19.1288	3-hydroxyacyl-CoA epimerase, required for fatty acid beta-oxidation; upregulated upon phagocytosis; transcription regulated by Mig1p, by white-opaque switching and by DNA methylation; transcriptional activation by oleate requires Ctf1p			1.6
	orf19.3924	Uncharacterized			1.6
<i>MTO1</i>	orf19.5050	Putative mitochondrial protein; transcription is upregulated in both intermediate and mature biofilms			1.6
<i>ABC1</i>	orf19.3331	Putative ubiquinol-cytochrome-c reductase; induced upon adherence to polystyrene; biofilm-induced			1.6

<i>CRP1</i>	orf19.4784	Plasma membrane copper transporter; CPx P1-type ATPase; mediates Cu resistance; similar to proteins of Menkes and Wilson disease; copper-induced; Tbf1p-activated; suppresses Cu sensitivity of <i>S. cerevisiae</i> cup1 mutant; biofilm-induced		1.6	
<i>RIM101</i>	orf19.7247	Transcription factor involved in alkaline pH response; required for alkaline-induced hyphal growth; role in virulence in mouse systemic infection; activated by C-terminal proteolytic cleavage; mediates both positive and negative regulation			1.6
<i>TSA1</i>	orf19.7398.1	Putative peroxidase; orf19.7398.1 is contig-truncated fragment of gene identical to TSA1; Tsa1p and Tsa1Bp role under oxidative/reductive stress, hyphal cell wall formation; in hyphal nucleus, cell wall; yeast-form nucleus, cytoplasm			1.6
<i>ADE13</i>	orf19.3870	Adenylosuccinate lyase; enzyme of adenine biosynthesis; soluble protein in hyphae; not induced during GCN response, in contrast to the <i>S. cerevisiae</i> ortholog; repressed by nitric oxide			1.6
	orf19.7594	Uncharacterized		1.6	
	orf19.5282	Hap43p-repressed gene; mRNA binds to She3p; decreased expression in hyphae compared to yeast-form cells; regulated by Efg1p and Efh1p; intron in 5'-UTR; transcriptionally activated by Mnl1p under weak acid stress		1.6	
	orf19.1580	Uncharacterized	-1.3	1.5	1.5
	orf19.5290	Hap43p-induced gene; transcription is negatively regulated by Sfu1p	-1.2	1.4	1.5
<i>LYS142</i>	orf19.4778	Putative transcription factor with zinc cluster DNA-binding motif; has similarity to <i>S. cerevisiae</i> Lys14p, which is a transcription factor involved in the regulation of lysine biosynthesis genes; fungal-specific	-1.3	1.7	1.4
<i>EHT1</i>	orf19.3040	Protein similar to <i>S. cerevisiae</i> Eht1p; transcription is induced in response to alpha pheromone in SpiderM medium		1.8	
	orf19.2739	Ortholog of <i>C. glabrata</i> Cagl0k02453gp			1.8
<i>INO1</i>	orf19.7585	Inositol-1-phosphate synthase; inositol biosynthesis; antigenic in human; repressed by farnesol in biofilm or by caspofungin; regulated during biofilm, planktonic growth; upstream inositol/choline regulatory element; glycosylation predicted	1.8		
	orf19.1117	Protein similar to <i>Candida boidinii</i> formate dehydrogenase; virulence-group-correlated expression; Hap43p-repressed gene	1.2		1.5
<i>UGA6</i>	orf19.5820	Putative GABA-specific permease; decreased transcription is observed upon benomyl treatment or in an azole-resistant strain that over expresses MDR1	1.2		1.5
	orf19.6682	Predicted ORF from Assembly 19; merged with orf19.1124.2 in Assembly 20	1.5	1.2	
	orf19.1124.2	<i>S. cerevisiae</i> ortholog Dph5p has diphthine synthase activity, has role in peptidyl-diphthamide biosynthetic process from peptidyl-histidine and localizes to cytoplasm			
	orf19.2076	Putative protein of unknown function; Hap43p-repressed gene; ortholog of <i>S. cerevisiae</i> YMR130W	1.2		1.5
	orf19.2509	Uncharacterized	1.2		1.5
<i>GCY1</i>	orf19.6757	Possible aldo/keto reductase; mutation confers hypersensitivity to toxic ergosterol analog; farnesol-downregulated; stationary phase enriched protein; biofilm- and planktonic growth-induced gene		1.2	1.5
<i>VPS24</i>	orf19.2031	Protein similar to <i>S. cerevisiae</i> Vps24p, which is a member of the ESCRT III protein sorting complex; downregulated upon adherence to polystyrene	1.2	1.6	
	orf19.7158	Protein of allantoate permease family; fungal-specific (no human or murine homolog); Hap43p-repressed gene		1.5	1.3

	orf19.6508	<i>S. cerevisiae</i> ortholog Lst7p has protein transporter activity, has role in intracellular protein transport, Golgi to plasma membrane transport and localizes to vesicle coat		1.2	1.5
	orf19.3572.3	Ortholog of <i>S. cerevisiae</i> Rpl31ap and Rpl31bp		1.5	1.3
	orf19.5093	Ortholog of <i>C. glabrata</i> Cagl0f08569gp		1.3	1.5
COX19	orf19.4967	Putative cytochrome c oxidase assembly protein; Plc1p-regulated	1.5	1.2	
	orf19.4450.1	Protein conserved among the fungal CTG-clade; gene contains two adjacent upstream SRE-1 elements; highly up-regulated in cecum-grown cells in a Cph2p-dependent manner; Hap43p-repressed gene; biofilm-induced	1.5	1.3	
IFF11	orf19.5399	Secreted protein required for normal cell wall structure and for virulence; member of the IFF family; Hap43p-repressed gene	1.3		1.6
	orf19.510	Uncharacterized		1.2	1.6
	orf19.4905	Hap43p-induced gene; repressed in a <i>ssr1</i> null mutant		1.3	1.5
TOP1	orf19.96	DNA topoisomerase I; required for wild-type growth and for wild-type mouse virulence; sensitive to camptothecin; induced upon adherence to polystyrene		1.5	1.3
RPS30	orf19.4375.1	Putative 40S ribosomal protein S30; shows colony morphology-related gene regulation by Ssn6p		1.5	1.3
	orf19.7304	Hap43p-induced gene; expression is upregulated early upon infection of reconstituted human epithelium (RHE), while expression of the <i>C. dubliniensis</i> ortholog is not upregulated; not required for viability		1.4	1.5
	orf19.2733	Putative subunit of phosphatidylinositol 3-kinase complexes I and II; transcription is activated in the presence of elevated CO ₂		1.4	1.5
	orf19.5390	Uncharacterized	1.3		1.6
ERG9	orf19.3616	Putative farnesyl-diphosphate farnesyl transferase (squalene synthase) involved in the sterol biosynthesis pathway; likely to be essential for growth; regulated by fluconazole and lovastatin; amphotericin B, caspofungin repressed		1.4	1.5
COI1	orf19.5063	Secreted protein; ciclopirox olamine induced; regulated by Ssn6p; induced by nitric oxide independent of Yhb1p; Hap43p-induced		1.4	1.5
	orf19.1041	Ortholog of <i>S. cerevisiae</i> Ctk2p		1.5	1.4
	orf19.4949	Uncharacterized	1.3	1.6	
	orf19.7310	Protein similar to <i>S. cerevisiae</i> Gin3p; transcription is upregulated in response to treatment with ciclopirox olamine; positively regulated by Sfu1p; Hog1p, fluconazole-downregulated; Hap43p-induced		1.4	1.6
	orf19.1029	<i>S. cerevisiae</i> ortholog Rpp1p has ribonuclease MRP activity, ribonuclease P activity and has role in intronic box C/D snoRNA processing, mRNA cleavage, rRNA processing, tRNA processing		1.5	1.4
	orf19.94	Uncharacterized	1.6	1.3	
	orf19.5474	Predicted ORF in Assemblies 19, 20 and 21; transcriptionally activated by Mnl1p under weak acid stress; transcription detected in high-resolution tiling array experiments		1.4	1.6
	orf19.3537	Putative sulfiredoxin; biofilm-induced gene; regulated by Tsa1p, Tsa1Bp in minimal media at 37 degrees C		1.5	1.4
	orf19.7239	Uncharacterized	1.5	1.5	

	orf19.470	Uncharacterized	1.6	1.4	
	orf19.3872	Ortholog of <i>S. cerevisiae</i> Ygr026wp		1.6	1.4
	orf19.4214	Protein not essential for viability		1.4	1.7
<i>SMC2</i>	orf19.3623	Protein similar to <i>S. cerevisiae</i> Smc2p, which is a component of the condensin complex involved in mitotic chromosome condensation; induced under hydroxyurea treatment		1.4	1.7
<i>LEU2</i>	orf19.7080	Isopropyl malate dehydrogenase; enzyme of leucine biosynthesis; upregulated in the presence of human whole blood or polymorphonuclear (PMN) cells; protein level decreases in stationary phase cultures		1.5	1.6
<i>ABP1</i>	orf19.2699	Protein similar to <i>S. cerevisiae</i> Abp1p, which is an actin-binding protein of the cortical actin cytoskeleton; caspofungin induced; protein only detected in stationary phase yeast-form cultures	1.5	1.7	
	orf19.1034	Hap43p-repressed gene; caspofungin repressed; biofilm- and planktonic growth-induced gene		1.7	1.5
<i>SNQ2</i>	orf19.5759	Protein similar to <i>S. cerevisiae</i> Snq2p transporter; member of PDR subfamily of ABC family; transposon mutation affects filamentation; benomyl-induced transcription; detected at yeast-form cell plasma membrane by mass spec		1.5	1.7
<i>PGA38</i>	orf19.2758	Putative GPI-anchored protein; adhesin-like protein; repressed during cell wall regeneration; possibly an essential gene, disruptants not obtained by UAU1 method		1.4	1.8
<i>LEM3</i>	orf19.3542	Putative membrane protein; early-stage biofilm-induced gene; mutation causes increased resistance to miltefosine		1.7	1.7
	orf19.2025	Uncharacterized		2.0	1.4
<i>GIT4</i>	orf19.1980	Putative glycerophosphoinositol permease; fungal-specific (no human or murine homolog); possibly an essential gene, disruptants not obtained by UAU1 method; Hap43p-repressed gene		2.0	1.6
<i>SCW11</i>	orf19.3893	Cell wall protein; transcription decreased in mutant lacking ACE2; downregulated in core caspofungin response; expression greater in high iron; possibly an essential gene, disruptants not obtained by UAU1 method; planktonic growth-induced		1.7	2.0
<i>HST6</i>	orf19.7440	ABC transporter related to mammalian P-glycoproteins; functional homolog of <i>S. cerevisiae</i> Ste6p (a-pheromone transporter); required for mating of MTLa cells; a-type specific, not regulated during white-opaque or yeast-hyphal switching	1.2	1.2	1.5
	orf19.3418	Uncharacterized	1.1	1.2	1.5
	orf19.1019	Predicted ORF from Assembly 19; removed from Assembly 20; merged with orf19.5718 in Assembly 21	1.1	1.5	1.4
	orf19.5718	Uncharacterized			
<i>GND1</i>	orf19.5024	Putative 6-phosphogluconate dehydrogenase; soluble in hyphae; farnesol-, macrophage-induced protein; antigenic in mice; protein present in exponential and stationary growth phase yeast cultures; Hap43p-induced	1.2	1.4	1.5
<i>SDC1</i>	orf19.3899	Putative COMPASS/SET1C histone methyltransferase complex subunit	1.2	1.3	1.5
	orf19.6184	Uncharacterized	1.2	1.4	1.6
<i>GPD1</i>	orf19.1756	Protein similar to <i>S. cerevisiae</i> glycerol-3-phosphate dehydrogenase (enzyme of glycerol biosynthesis); biofilm-induced expression; regulated by Efg1p; regulated by Tsa1p, Tsa1Bp under H ₂ O ₂ stress conditions	1.2	1.4	1.5
	orf19.5574	<i>S. cerevisiae</i> ortholog Ygr012wp localizes to mitochondrial outer membrane	1.3	1.3	1.6

<i>SMT3</i>	orf19.670	SUMO, small ubiquitin-like protein; Smt3p-conjugated proteins localize to septation site and mother side of bud neck; <i>C. albicans</i> septins appear not to be Smt3p-modified, in contrast to <i>S. cerevisiae</i> septins	1.6	1.2	1.4
<i>IFE2</i>	orf19.5288	Putative alcohol dehydrogenase; decreased expression in hyphae compared to yeast-form cells; Efg1p-regulated; fluconazole-induced; Hog1p-induced; increased expression in response to prostaglandins		2.4	1.8
	orf19.1975	<i>S. cerevisiae</i> ortholog Dib1p has role in nuclear mRNA splicing, via spliceosome and localizes to U5 snRNP, U4/U6 x U5 tri-snRNP complex	1.1	1.8	1.4
	orf19.4612	Hap43p-repressed gene	1.2	1.5	1.7
	orf19.5952	Hap43p-induced protein of unknown function; induced by nitric oxide independent of Yhb1p	1.4	1.3	1.7
	orf19.4706	Planktonic growth-induced gene; greater mRNA up-regulated in a <i>cyr1</i> or <i>ras1</i> homozygous mutant than in wild type; fluconazole-induced; transcription is induced in response to alpha pheromone in SpiderM medium	1.7	1.5	1.6
<i>SSY1</i>	orf19.814	Amino acid sensor, required for wild-type hyphal growth on solid serum or Lees media, not for hyphal growth under all conditions; 12 predicted membrane spanning regions; Hap43p-repressed gene	1.2	2.0	2.1
<i>HYM1</i>	orf19.796	Mo25 family domain protein of RAM cell wall integrity signalling network; role in cell separation, azole sensitivity; required for hyphal growth	1.3	2.0	2.2

* According to the *Candida* genome database (www.candidagenome.org)

Positive fold change indicates up-regulated genes and negative fold change down-regulated genes. Fold change values are relative to untreated sample at the same time point.

GENERAL DISCUSSION

General discussion

Oxylipins are widely distributed in nature, but only a small percentage of the fungal kingdom has been studied. In this study we identified and extended the production of prostaglandins (PGE₂ & PGF_{2α}) from exogenous arachidonic acid (AA) [20:4(n-6)] by *Candida* spp. to *C. dubliniensis*. It is now believed that almost all fungi, has the ability to produce eicosanoids from exogenous AA. However, the exact reason why these eicosanoids are produced under certain conditions is still a matter of speculation.

The production of PGE₂ by *C. albicans* is well known and extensively studied, especially due to its role during infection and the host immune response (Alem & Douglas, 2004, 2005; Erb-Downward & Noverr, 2007; Noverr et al., 2001). Despite all these studies, the exact mechanism of how these prostaglandins are produced is still lacking. This study tried to identify possible enzymes involved in this pathway, through the use of different known arachidonic acid metabolism inhibitors, such as cytochrome P450 (CYP450), multicopper oxidase, cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors. This demonstrated that cytochrome P450s and multicopper oxidases are involved in this pathway in both *C. albicans* and *C. dubliniensis* biofilms. Similarly, Erb-Downward and Noverr (2007) identified a multicopper oxidase or laccase homologue (Fet3p) to be involved in PGE₂ production by planktonic *C. albicans* cells. We also speculated that these enzymes are not the only enzymes involved due to the inability to completely inhibit PGE₂ production. It is also clear that mammals and fungi employ different eicosanoid biosynthesis pathways. This indicates that a further detailed analyses needs to be done in this regards using a combination of inhibitors assessing the production of not only PGE₂ but other oxylipins, as well and including other pathogenic *Candida* species.

The analysis of global gene expression in *C. albicans* in the presence of AA, did not indicate any genes directly involved in PGE₂ production. This indicates the complex mechanisms involved during AA metabolism. It would also be helpful to evaluate gene expression during earlier time points (e.g. 1 h) and to include the non-methylene interrupted (NMIFA) n-6 fatty acid, sciadonic acid (SA) [20:3(n-6)] in these

experiments. The comparison of the transcriptomes of the cells during an infection in the presence of AA and SA, would give a more detailed evaluation of the complex mechanisms involved during an infection. The identified genes in this study or homologues of these genes, will need to be evaluated in other prostaglandin producing fungi as well as the use of other prostaglandin fatty acid precursors.

The overproduction of PGE₂ during an infection may be harmful to the host and beneficial to the pathogen (Betz & Fox, 1991; Navarathna et al., 2007; Romani, 2000). This could also affect Th1 and Th2 responses of the host immune system, with an imbalance in these responses leading to detrimental effects to the host, such as serious autoimmune diseases (Shibata et al., 2005; van der Pouw Kraan et al., 1995). In addition, the ability of *C. albicans*, to utilize PGE₂ as an important virulence factor (Erb-Downward & Noverr, 2007), contributes to the importance to control the production during an infection, especially due to the increase in immunocompromized individuals (Low & Rotstein, 2011). This together with the increased ability of fungal pathogens to gain resistance towards known antifungals (Low & Rotstein, 2011), urged us to try and develop a system to decrease PGE₂ production during *C. albicans* and *C. dubliniensis* infections. This was attempted by the use of a NMIFA, SA. The main difference between these fatty acids is the absence of the $\Delta 8$ -ethylenic bond in SA. Interestingly, this fatty acid competes with AA for incorporation into the phospholipids of mammalian cells, but cannot be directly metabolized to produce prostaglandins (Berger et al., 2002; Berger & Jomard, 2001; Tanaka et al., 2001). This suggests that this bond is not necessary for acylation into phospholipids, but for prostaglandin production. This led to the hypothesis that production of PGE₂ and inflammatory cytokines during *Candida* infection can be modulated by incorporation of SA into the host cellular lipids.

This study confirms the competition of SA with AA for the incorporation into the phospholipids of the epithelial cells. Interestingly, PGE₂ production was decreased in the presence of SA during infection with *C. albicans* and *C. dubliniensis*, as well as an alteration in the cytokine profiles. This could shift the immune response to benefit the host. Similar to the epithelial cells, these *Candida* species was unable to produce PGE₂ from SA, despite the structural similarity between AA and SA. This indicates the importance of the $\Delta 8$ -ethylenic bond in AA

during PGE₂ production, not only on mammalian cells, but also in fungi. It would be interesting to see what effect supplementing AA and SA together have on PGE₂ and cytokine production. It is speculated that the available AA would be metabolized first followed by SA, leading to an initial increase in PGE₂ followed by decrease in this production.

The study indicated that by changing the lipid profile of infected epithelial cells, the immune response can be altered towards a beneficial one for the host. However, a further detailed analysis is needed to evaluate the effect of SA supplementation during a *Candida* infection on the immune response by using other cell types, such as macrophages, endothelial cells etc., followed by *in vivo* studies. The approach of changing the lipid composition in susceptible hosts is very feasible and can be done by increasing these fatty acids in their dietary consumption, as has been done by supplementing milk with n-3 polyunsaturated fatty acids (PUFA) to reduce the risk of cardiovascular diseases (German et al., 1995; Lopez-Huertas, 2010).

References

- Alem MAS & Douglas LJ. (2004). Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans*. *Antimicrob Agents Chemother* 48: 41-47.
- Alem MAS & Douglas LJ. (2005). Prostaglandin production during growth of *Candida albicans* biofilms. *J Med Microbiol* 54: 1001-1005.
- Berger A & Jomard A. (2001). Fatty acid uninterrupted by a methylene as anti-inflammatory agents in superficial tissues of mammals. Patent no. US 6,280,755 B1.
- Berger A, Monnard I, Baur M, Charbonnet C, Safonova I, Jomard A. (2002). Epidermal anti-inflammatory properties of 5,11,14 20:3: Effects on mouse ear edema, PGE₂ levels in cultured keratinocytes, and PPAR activation. *Lipids Health Dis* 1: 1-12.

-
- Betz M & Fox BS. (1991). Prostaglandin E₂ inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J Immunol* 146: 108-113.
- Erb-Downward JR & Noverr MC. (2007). Characterization of prostaglandin E₂ production by *Candida albicans*. *Infect Immun* 75: 3498-3505.
- German JB, Gershwin ME & Berger A. (1995). Non-methylene interrupted fatty acids as immunomodulators. Patent no. 5,456,912.
- Lopez-Huertas E. (2010). Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. *Pharmacol Res* 61: 200-207.
- Low C-H & Rotstein C. (2011). Emerging fungal infections in immunocompromised patients. *F1000 Medicine Reports* 3: 14-21.
- Navarathna DHMLP, Nickerson KW, Duhamel GE, Jerrels TR & Petro TM. (2007). Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. *Infect Immun* 75: 4006-4011.
- Noverr MC, Phare SM, Toews GB, Coffey MJ & Huffnagle GB. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infect Immun* 69: 2957-2963.
- Romani L. (2000). Innate and adaptive immunity in *Candida albicans* infections and saprophytism. *J Leukoc Biol* 68: 175-179.
- Tanaka T, Morishige J, Takimoto T, Takai Y & Satouchi K. (2001). Metabolic characterization of sciadonic acid as an effective substitute for arachidonate of phosphatidylinositol. *Eur J Biochem* 268: 4928-4939.

SUMMARY / OPSOMMING

Summary

Most of what is known about the biology and function of oxylipins, oxygenated polyunsaturated fatty acids and metabolites, including the eicosanoids such as prostaglandins, comes from the study of mammalian biology. These compounds are ubiquitous in nature and found in all eukaryotic organisms, including the fungal domain. It is also in this group of organisms that the least is known about the metabolic pathways leading to the production of oxylipins, including those derived from arachidonic acid (AA) (n-6 fatty acid), and the functions of these compounds in the biology of fungi and yeasts. *Candida* species has the ability to produce pro-inflammatory eicosanoids, such as prostaglandin E₂ (PGE₂), from host derived AA. *Candida albicans* is an important opportunistic pathogen in humans causing systemic infections. An important virulence factor in *C. albicans* is the ability to produce pro-inflammatory PGE₂, which enhances biofilm formation and influences host immune responses. Biofilms increase damage in host cells and are more resistant to antifungal drugs than planktonic yeast cells. This is an important area of research which may aid in the understanding of the complex interactions between host and pathogen, leading to the identification of novel antifungals or drug targets. This study evaluated the production of the prostaglandins, PGE₂ and PGF_{2α}, from exogenous AA, by biofilms of *C. albicans* and the closely related *C. dubliniensis* as well as the effect of different AA metabolism inhibitors on PGE₂ production. *Candida albicans* and *C. dubliniensis* biofilms were both capable of producing PGE₂ and PGF_{2α}, from exogenous AA. The use of different inhibitors suggested that cytochrome P450s and multicopper oxidases are involved in PGE₂ production by these *Candida* biofilms. It is known that mammalian cells cannot produce PGE₂ from non-methylene interrupted fatty acids (NMIFAs), such as sciadonic acid (SA) (n-6 fatty acid). This property provides these fatty acids with potential anti-inflammatory activities. This study indicated the incorporation of SA into the lipids of epithelial cells, which reduced PGE₂ production and influenced cytokine profiles in SA supplemented epithelial cells infected with *C. albicans* or *C. dubliniensis*. This suggest that the incorporation of n-6 NMIFAs, such as SA, might lead to a reduction in pro-inflammatory prostaglandins, especially PGE₂, which could benefit the host during a *Candida* infection. Interestingly, both *C. albicans* and *C. dubliniensis* biofilms were unable to produce PGE₂ from exogenous SA. Further genomic

hybridization studies were used to evaluate the regulation of *C. albicans* biofilm genes during incubation in the presence of exogenous AA and SA. Transcriptional analysis indicated that the genes differentially expressed in the presence of AA had diverse functions not normally required for cell growth. Genes encoding for oxidoreductase and hydrolase activity were regulated, but were not clearly involved in PGE₂ synthesis. Interestingly, genes that encode for ABC transporters, as well as genes associated with filamentous and hyphal growth, carbohydrate metabolic processes and oxidative stress response were differentially expressed by the presence of AA. Further studies of these differentially expressed genes are needed to evaluate how they may be involved in AA metabolism and PGE₂ production.

Keywords: Biofilms; *Candida albicans*; *Candida dubliniensis*; Eicosanoids; ELISA; Hep2C; Non-methylene interrupted fatty acids; Oxidoreductases; Polyunsaturated fatty acids; Prostaglandins

Opsomming

Meeste van wat bekend is oor die biologie en die funksie van oksilipiene, suurstofryke poli-onversadigde vetsure en metaboliete, insluitende die eikosanoïede soos prostaglandiene, kom vanuit die studie van soogdierbiologie. Hierdie verbindings is alomteenwoordig in die natuur en kom voor in alle eukariotiese organismes, insluitende fungi. Dit is ook in hierdie groep organismes wat die minste bekend is oor die metaboliese bane wat lei tot die produksie van oksilipiene, insluitend die afgelei van arachidoonsuur (AA) (n-6 vetsuur), en die funksies van hierdie verbindings in die biologie van fungi en giste. *Candida* spesies het die vermoë om pro-inflammatoriese eikosanoïede, soos prostaglandien E_2 (PGE_2), te produseer vanaf AA wat deur die gasheer vrygestel word. *Candida albicans* is 'n belangrike opportunistiese patoëen wat sistemiese infeksies in mense veroorsaak. 'n Belangrike virulensiefaktor in *C. albicans* is die vermoë om pro-inflammatoriese PGE_2 , wat biofilm vorming verhoog en die gasheer se immuunstelsel beïnvloed, te produseer. Biofilms verhoog die skade aan gasheerselle en is meer bestand teen antifungale middels as planktoniese gisselle. Dit is 'n belangrike navorsingsveld wat kan bydra tot die begrip van die komplekse interaksies tussen gasheer en patoëen en wat kan lei tot die identifisering van nuwe antifungale middels of teikens. Hierdie studie het die produksie van die prostaglandiene, PGE_2 en $PGF_{2\alpha}$, vanaf eksterne AA, deur biofilms van *C. albicans* en die nabyverwante gisspesies, *C. dubliniensis*, sowel as die effek van verskillende AA metabolisme inhibeerders op PGE_2 produksie bepaal. *Candida albicans* en *C. dubliniensis* biofilms was albei in staat om PGE_2 en $PGF_{2\alpha}$ vanaf eksterne AA te vervaardig. Die gebruik van verskillende inhibeerders het aangedui dat sitochroom P450s en multikoperoksidases betrokke is in PGE_2 produksie deur hierdie *Candida* biofilms. Dit is bekend dat soogdierselle nie PGE_2 kan produseer vanaf nie-metiel onderbreekte vetsure, soos skiadoonsuur (SA) (n-6 vetsuur) nie. Hierdie eienskap verleen aan hierdie vetsure moontlike anti-inflammatoriese aktiwiteite. Hierdie studie het die inkorporasie van SA in die lipiede van epiteelselle aangedui. Dit het PGE_2 produksie verminder en sitokienprofiel beïnvloed in epiteelselle wat SA geïnkorporeer het en geïnfekteer is met *C. albicans* of *C. dubliniensis*. Dit dui daarop dat die inkorporering van n-6 nie-metiel onderbreekte vetsure, soos SA, kan lei tot 'n afname in die pro-inflammatoriese prostaglandiene, veral PGE_2 , wat tot voordeel van die gasheer tydens 'n *Candida*-

infeksie kan wees. Interessant genoeg was beide *C. albicans* en *C. dubliniensis* biofilms nie in staat om PGE₂ te produseer vanaf eksterne SA nie. Verdere genomiese hibridiseringstudies is gebruik om die regulering van *C. albicans* biofilm gene tydens inkubasie in die teenwoordigheid van eksterne AA en SA te evalueer. Transkripsionele analise het aangedui dat die gene wat differensieël uitgedruk is in die teenwoordigheid van AA uiteenlopende funksies het wat nie normaalweg vereis word vir selgroeï nie. Gene wat kodeer vir oksidoreduktase en hidrolase aktiwiteit was gereguleer, maar is nie duidelik betrokke by PGE₂ produksie nie. Interessant genoeg, gene wat kodeer vir ABC-transporters, asook gene wat betrokke is by filamentagtige en hifese groei, koolhidraat metaboliese prosesse en oksidatiewe stres reaksie, is differensieël uitgedruk in die teenwoordigheid van AA. Verdere studie van hierdie differensieël uitgedrukte gene is nodig om te evalueer hoe hulle betrokke mag wees in AA metabolisme en PGE₂ produksie.

Sleutelwoorde: Biofilms; *Candida albicans*; *Candida dubliniensis*; Eikosanoïede; ELISA; Hep2C; Nie-metiel onderbreekte vetsure; Oksidoreduktase, Poli-onversadigde vetsure; Prostaglandiene