Effect of fatty acids on biofilm formation, oxidative stress and antifungal susceptibility of *Candida albicans* and *Candida dubliniensis*

By

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GENERAL DISCUSSION

SUMMARY AND KEYWORDS

OPSOMMING EN SLEUTELWOORDE

Chapter 1

Literature review

1.1. Motivation

Candida albicans and *C. dubliniensis* are dimorphic yeasts, able to grow both as yeasts and mycelia. *Candida albicans* and *C. dubliniensis* are closely related species and exist as commensals of the gastrointestinal and genitourinary tract in healthy individuals [Molero *et al*., 1998; Ramage *et al*., 2001; Berman & Sudbery, 2002]. However, in individuals who are immune compromised due to HIV infection or those using immune suppressants, they are known to cause Candidemia and Candidiasis [Coleman *et al*., 1997]. Biofilm formation is a major virulence factor in the pathogenicity of these species, partly due to their increased resistance to antifungal supplementation [Ballie & Douglas, 1999]. Commonly used antifungal compounds include polyenes and azoles; however *Candida* species have developed resistance towards them in recent years [Al-Mohsen & Hughes, 1998].

Fatty acids have long been known to have antifungal properties and their use as antifungal agents offer some advantages over conventional antifungal compounds currently in use [Liu *et al*., 2008]. The most important target of conventional antifungal supplementation includes the fungal membrane. Similarly, antifungal fatty acids are also known to target the fungal membrane [Avis & Bélanger, 2001]. They are naturally taken up and incorporated into the fungal membranes, where they increase the unsaturation index, increasing membrane fluidity, leading to membrane disruption and leakage of cellular material [Avis, 2007]. In addition, an increase in unsaturation of membranes also increases fatty acid peroxidation, making cells more susceptible to oxidative stress [Holman, 1954; Cortés-Rojo, 2009; Ferreira *et al*., 2011]. The antifungal efficiency of these fatty acids increases with chain length and unsaturation. Understanding the effect

that fatty acids have on the mitochondria and oxidative stress may be useful in finding alternative agents that can be used to combat fungal infections.

1.2. Introduction

Oxidative stress is a phenomenon associated with the action of free radicals and reactive metabolites in living organisms. It is associated with pathogenic mechanisms of several human diseases such as atherosclerosis and cardiovascular diseases [Ballinger, 2005], neurodegenerative diseases [Aruoma *et al.,* 2007] as well as the aging process in all organisms [Hulbert, 2005]. Oxidative stress is generally referred to as an imbalance between reactive oxygen species (ROS), which are oxidants, and the antioxidant defence system. It is defined as a disturbance between oxidants and antioxidants in favour of the oxidants, leading to potential damage [Sies, 1997]. Such damage is often termed oxidative damage and is defined as the biomolecular damage caused by attack of ROS upon constituents of living organisms [Halliwell & Whiteman, 2004].

Normally, the production of oxidants is approximately balanced with the antioxidant defence system [Halliwell & Gutteridge, 2007]. However, the balance is not always perfect and the antioxidants will mainly control the levels of oxidants rather than eliminate them, since under physiological conditions complete suppression of the oxidants will not be beneficial [Ďuračková *et al.,* 1999; Pomposiello & Demple, 2002]. Reactive oxygen species are beneficial for cell proliferation and have gained attention as important chemical mediators that regulate signal transduction [Nakamura, 1997;

Suzuki *et al.,* 1997]. Furthermore, inflammatory cells including neutrophils, eosinophils and phagocytes also contribute to cellular ROS build-up [Klauning *et al*., 2010]. Phagocyte ROS production through NADPH oxidase, an enzyme that catalyses the one electron reduction of oxygen (O_2) to $O_2^{\bullet-}$, involves activation by a variety of endogenous and exogenous stimuli which allow phagocytes to undergo a respiratory burst leading to transient increase in O_2 uptake. The ROS produced act as cellular defence mechanisms against invading pathogens [McCord, 2000; Halliwell, 2006; Klauning *et al*., 2010].

The mitochondrial electron transport chain consumes O_2 during cellular respiration and about 4-5% of O_2 consumed is converted to ROS [St-Pierre *et al.,* 2002]. These ROS (Table 1), include both oxygen-centred radicals, such as the superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$) and the non-radical derivatives of O_2, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [Halliwell & Chirico, 1993].

These ROS are highly reactive and can damage the cell by radically attacking DNA, proteins and membrane fatty acids (lipid peroxidation) and therefore result in the formation of other reactive metabolites. Furthermore, the accumulation of these oxidised reactive metabolites in the cell will eventually lead to cell death [Ott *et al.,* 2007]. Because the cell is continuously exposed to various conditions of oxidative stress from numerous sources, this has led to the development of the cells defence system to protect itself against damaging effects of excessive production and accumulation of the ROS and other reactive metabolites [Ďuračková, 1998]. The system is organised on three levels, the antioxidant defence system, non-enzyme antioxidant system and the cell repair system [Galbusera *et al*., 2006]. The antioxidant defence system (high molecular-weight antioxidants) is a direct approach and involves systems preventing

free radical formation. The non-enzyme antioxidant defence system (low molecular-weight antioxidants) is an indirect approach that involves systems eliminating previously formed ROS by scavenging and trapping them into non-radical or non-toxic metabolites [Li *et al*., 2009]. The cell repair system is responsible for recognition and decomposition of impaired molecules and consists of the enzymes proteinases, which repair oxidatively damaged proteins, lipases, against oxidatively damaged lipids, and the DNA repair system, repairing modified DNA bases [Ďuračková, 1998; 2010].

Table 1: Radical and non-radical oxygen metabolites.

Radicals	Non-Radicals
Superoxide ($O_2^{\bullet -}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($HO^{\bullet}$)	Singlet oxygen ($^1O_2$)
Alkoxyl ($RO^{\bullet}$)	Hypochlorous acid (HOCL)
Peroxyl ($ROO^{\bullet}$)	Ozone (O_3)
Nitric oxide ($NO^{\bullet}$)	Peroxynitrite (ONOOH)

1.3. Reactive oxygen species production

1.3.1. Mitochondrial ROS production

During normal aerobic metabolic activity, O_2 is continuously being reduced to water (H_2O) by the electron transport chain (ETC) leading to the generation of ROS at steady state concentration [Dröge, 2002]. The $O_2^{\bullet-}$ radical is the primary ROS generated by mitochondria and it is formed by monoelectronic reduction of O_2 [Fridovich, 1995; Halliwel, 1995; Kowaltowski *et al*., 2009]. It is mainly generated at complex I and III, with complex I producing most of the $O_2^{\bullet-}$ from leakage of electrons between the two complexes [Kussmaul & Hirst, 2006; Hirst *et al*., 2008]. Complex I (NADH-ubiquinone oxidoreductase) is an integral inner membrane multi-protein complex exposed to both matrix and intermembrane space and an entry point for electrons from NADH into the ETC. A flavin mononucleotide (FMN) cofactor accepts electrons from NADH and passes them through a chain of seven iron-sulfur (Fe-S) centres to the coenzyme Q (CoQ) reduction site [Sazanov, 2007]. The formation of $O_2^{\bullet-}$ will be as a result of leakage of electrons to O_2 in the complex and the production sites are located between flavin and rotenone-binding sites. Complex I is thought to produce $O_2^{\bullet-}$ through processes of reverse electron transfer (RET) and forward electron transfer (FET) (Fig. 1). Forward electron transport involves the transfer of electrons in the ETC from NADH to ubiquinol (QH_2) with the formation of $O_2^{\bullet-}$ from leakage of electrons to O_2. Reverse electron transport is a set of reactions in the ETC that allows electrons to be transferred against the gradient of redox potentials of electron carriers, from reduced CoQ to NAD^+ with some electrons leaking to O_2. The tricarboxylic acid cycle (TCA) provides electrons

through succinate to allow this thermodynamically unfavourable process to proceed [Andreyev *et al.,* 2005; Murphy, 2009].

Complex III (ubiquinol-cytochrome c reductase) is an enzyme complex oxidizing QH_2 using cytochrome c (cyt c) as electron acceptor. The oxidation of QH_2 proceeds in a set of reactions known as the Q-cycle (Fig. 2) in which QH_2 transfers one electron to O_2 via Fe-S protein and cyt c_1 producing H_2O as the final product [Turrens, 2003]. The resulting ubisemiquinone ($Q^{\bullet}_o$), formed at the Q_o site, is unstable and donates the second electron to cytochrome b (cyt b) which serves as a path to provide electrons to the Q_i site. The Q_o site oxidizes two QH_2 molecules and provides two electrons, the first electron at the Q_i site generates a stable semiquinone ($Q^{\bullet}_i$) that is reduced to a QH_2 by the second electron but eventually electrons leak at either the Q_o and Q_i sites to O_2, producing $O_2^{\bullet-}$.

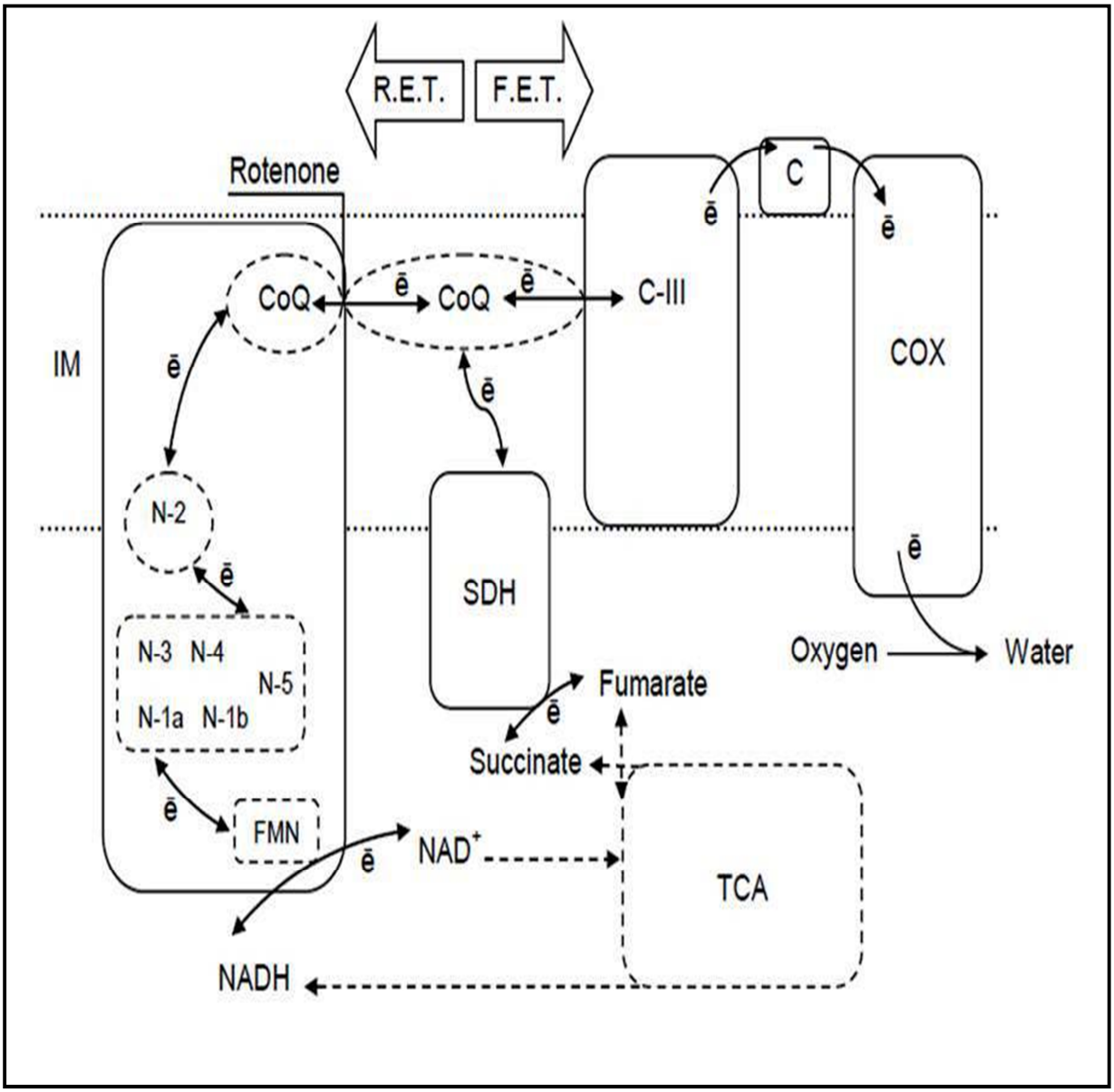

Fig. 1: Complex I produces most of the $O_2^{\bullet-}$ from leakage of electrons through the reverse electron transport (RET) and forward electron transport (FET). Abbreviations: IM (inner mitochondrial membrane); TCA (tricarboxylic acid cycle); SDH (succinate dehydrogenase); C-III (complex III); *c* (cytochrome *c*); COX (cytochrome *c* oxidase); FMN (flavin mononucleotide); N-1a, N-1b, N-2, N-3, N-4, N-5 (iron-sulphur centres of complex I); CoQ (coenzyme Q); NAD^+ (nicotinamide adenine dinucleotide); NADH (reduced nicotinamide adenine dinucleotide) (Andreyev *et al*., 2005).

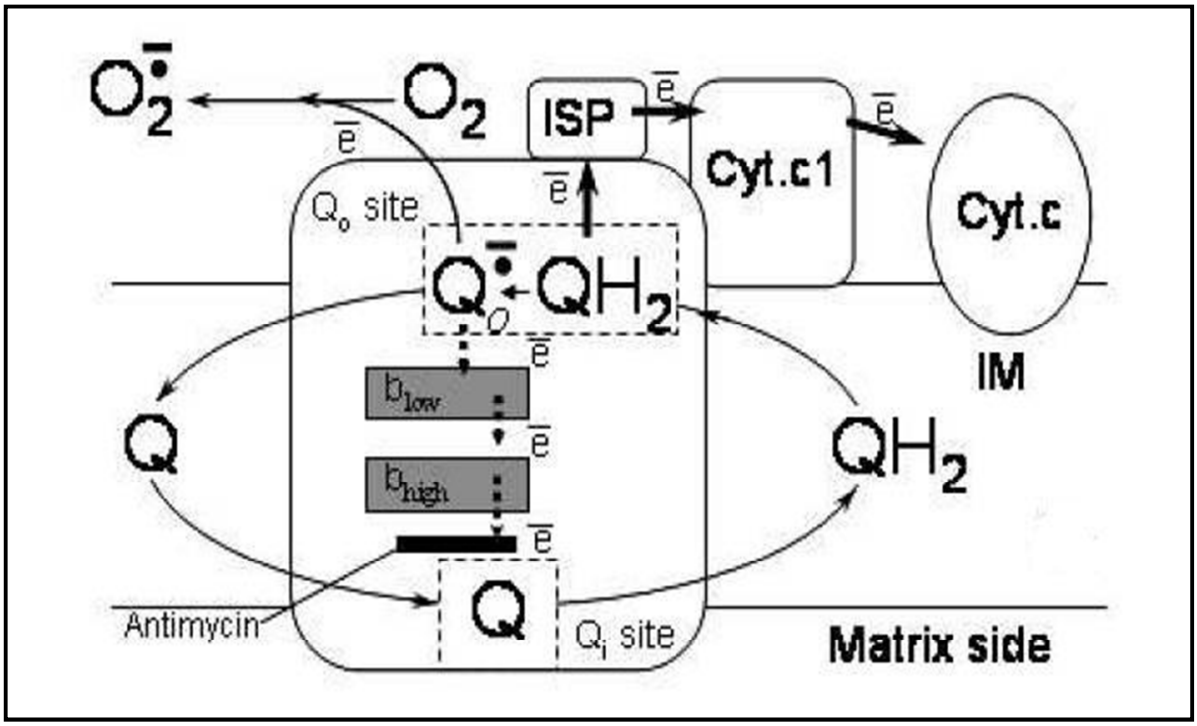

Fig. 2: The Q-cycle model of coenzyme Q oxidation. Superoxide anion production at complex III. Abbreviations: ubiquinol (QH_2); ubisemiquinone ($Q^{\bullet}_o$); semiquinone ($Q^{\bullet}_i$); ISP (iron-sulphur proteins); IM (inner mitochondrial membrane); cyt *c* (cytochrome *c*); cyt *c*1 (cytochrome *c*1) (Andreyev *et al*., 2005).

The $O_2^{\bullet-}$ produced is dismutated to H_2O_2, which is relatively stable [Bienert *et al.,* 2006], by copper-zinc superoxide (CuZnSOD) in the intermembrane space and by manganese superoxide (MnSOD) in the mitochondrial matrix [Weisiger & Fridovich, 1973]. The $HO^{\bullet}$, which is a highly reactive species that reacts quickly with almost every biological molecule, can be generated from the oxidation of H_2O_2 in the presence of a transition metal such as ferrous iron (Fe^{2+}) through the Fenton's reaction. The reaction is simultaneous with the subsequent interaction of $O_2^{\bullet-}$ with ferric ion (Fe^{3+}) in the Haber-Weiss reaction for the regeneration of the Fe^{2+} necessary for the generation of $HO^{\bullet}$ (Fig. 3) [Halliwell & Gutteridge, 1990; Temple *et al*., 2005].

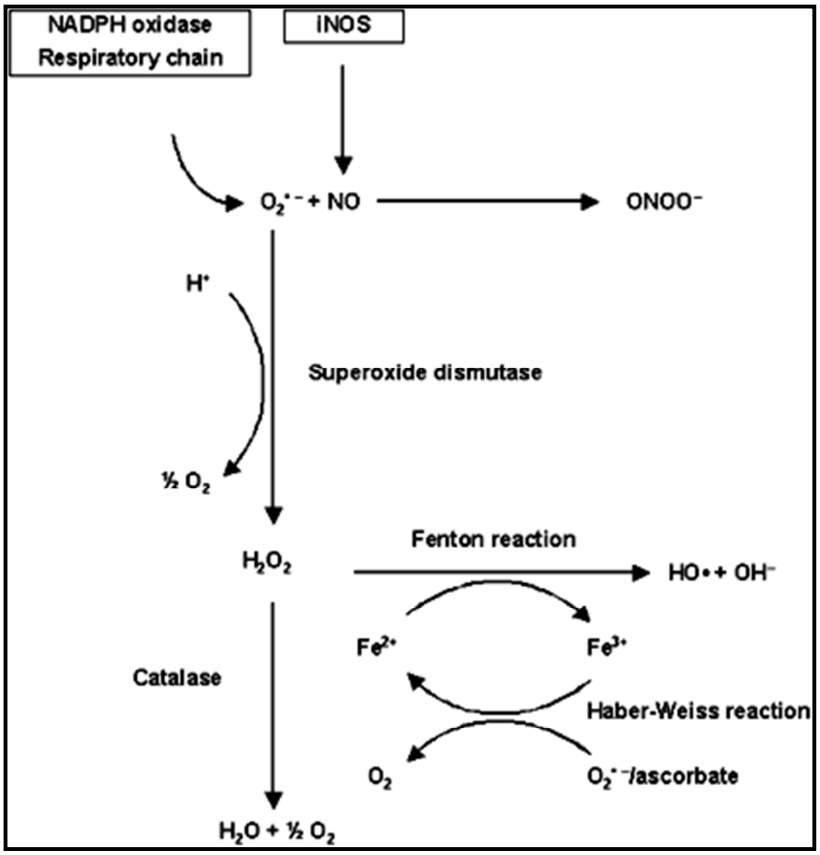

Fig. 3: Reactive oxygen species produced by different reactions and the antioxidant defence system. Abbreviations: inducible nitric oxide synthase (INOS); nitric oxide (NO); peroxynitrite ($ONOO^-$); reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Van Meeteren *et al*., 2005).

1.3.2. Other ROS production sites

Reactive oxygen species can also be produced at other sites except the mitochondria, such as peroxisomes [Klaunig & Kamendulis, 2004]. Peroxisomes are organelles that consume oxygen and therefore contribute to the cellular ROS that are generated in the cell. The production of ROS in the peroxisomes involves peroxisomal oxidases including acyl-CoA oxidase and xanthine oxidase, which generate H_2O_2 and superoxide radicals [Schrader & Fahimi, 2006].

1.4. Oxidative stress

1.4.1. Oxidative damage of cell constituents

1.4.1.1. Mitochondrial DNA

Since the mitochondrion is the main site of ROS production, mitochondrial DNA (mtDNA) is an important target for oxidative damage [Cadenas & Davies, 2000]. Oxidative damage to mtDNA could lead to lethal cell injury through the disruption of the ETC, mitochondrial membrane potential and ATP generation [Ott *et al.,* 2007]. ROS-induced mtDNA damage is due to attack on purine/pyrimide bases and deoxyribose/ribose sugars and can result in single or double-strand breakage, as well as DNA cross-links [Bohr, 2002; Cooke *et al.,* 2003]. The $HO^{\bullet}$ is the predominant ROS that targets mtDNA while $O_2^{\bullet -}$ and H_2O_2 are less reactive [Guyton & Kensler, 1993; Barber & Harris, 1994; Lu *et al.,* 2001]. The accumulation of 8-hydroxydeoxyguanosine (Fig. 4), a product of $HO^{\bullet}$ attack on deoxyguanosine, is widely used as the biomarker of oxidatively damaged DNA and is probably a major source of mitochondrial genomic instability, leading to respiratory dysfunction [Giulivi *et al.,* 1995; Hwang & Bowen, 2007].

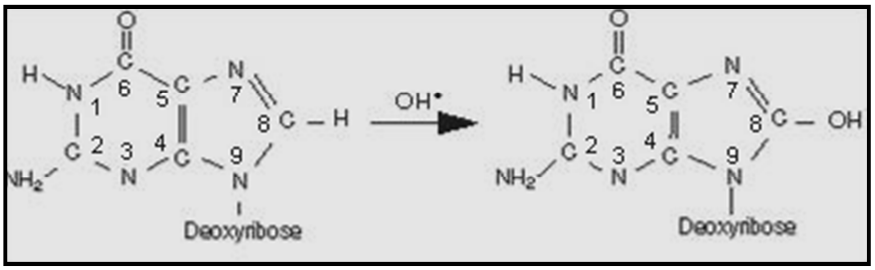

Fig. 4: Formation of 8-hydroxydeoxyguanosine- a biomarker of oxidatively damaged DNA.

1.4.1.2. Mitochondrial proteins

Proteins that are present in the mitochondria are susceptible to oxidative damage due to their close proximity to ROS production sites. Reactive oxygen species, specifically $O_2^{\bullet-}$, directly oxidize and inactivate the iron sulfur centre $[4Fe\text{-}4S]^{+2}$ of proteins such as aconitase to form the inactive $[3Fe\text{-}4S]^{+}$ sulphur centre, followed by the release of Fe^{2+} and also the carboxylation and degradation of the enzyme (Fig. 5) [Fridovich, 1997]. The released Fe^{2+} can participate in the subsequent interaction with H_2O_2 through the Fenton's reaction and result in the generation of the potent $HO^{\bullet}$, which can further oxidize mitochondrial proteins, mtDNA and membrane fatty acids [Buettner & Schafer, 2000; Temple *et al.,* 2005]. The Fe-S centres in aconitase are selectively vulnerable to ROS and it has been found that aconitase is the most ROS-sensitive yeast enzyme [Lushchak, 2010] and that mammalian aconitase-2 (Aco-2) is specifically targeted by oxidative damage during aging [Yan *et al.,* 1997; Tortora *et al.,* 2007]. Aconitase-2 is a mitochondrial enzyme that plays an important role in the Krebs cycle, catalyzing the conversion of citrate to isocitrate and inactivation can result in Krebs cycle dysfunction, inhibiting energy production and cell viability and possibly result in the cell aging, eventually leading to cell death [Bulteau *et al.,* 2003].

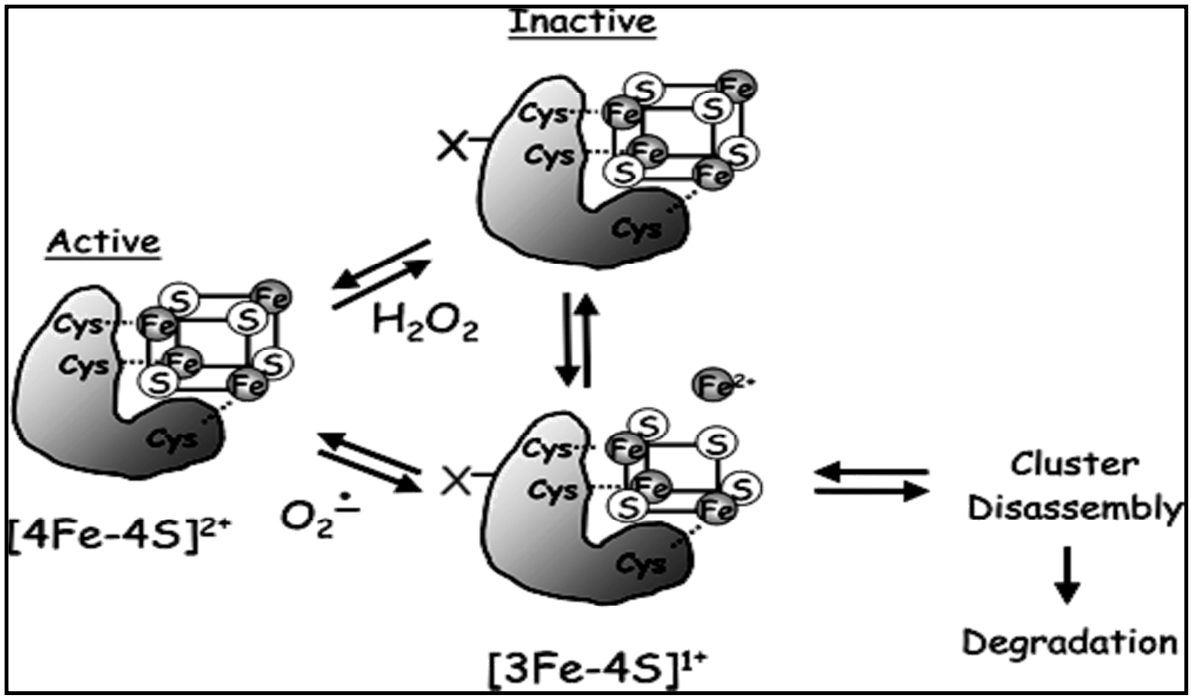

Fig. 5: Intact mitochondrial aconitase undergoes H_2O_2 and $O_2^{\bullet-}$ mediated inactivation. Inactivation precedes release of Fe^{2+} from the $[4Fe\text{-}4S]^{2+}$ cluster present in the active site of the enzyme (Bulteau *et al*., 2003).

1.4.1.3. Lipid peroxidation

Mitochondrial membrane phospholipids are located at the primary site of ROS production and because of this close proximity they are susceptible to oxidative damage. Membrane phospholipids have a high content of polyunsaturated fatty acids (PUFAs) [Hulbert, 2005], which are long fatty acid chains with more than one double bond. Polyunsaturated fatty acids have the highest sensitivity to oxidative damage and sensitivity increases as a power function of the number of double bonds per fatty acid chain [Enoch *et al.,* 1976; Wagner *et al.,* 1994; Pamplona *et al.,* 1998]. The oxidation of membrane fatty acids leads to a vicious cycle that when initiated is not easy to bring to

an end. These oxidized fatty acids form lipid peroxides which lead to an increase in radically reactive metabolites. The degree at which these reactive metabolites are formed will depend on membrane fatty acids composition, specifically their different susceptibilities to oxidation [Holman, 1954]. Membranes with large amounts of highly PUFAs will have a large feedback influence on the formation of reactive lipid radicals while membranes with low content of PUFAs, such as those of yeasts grown without exogenous PUFAs, will only have a small influence (Fig. 6) [Cortés-Rojo *et al.,* 2009; Lushchak, 2010].

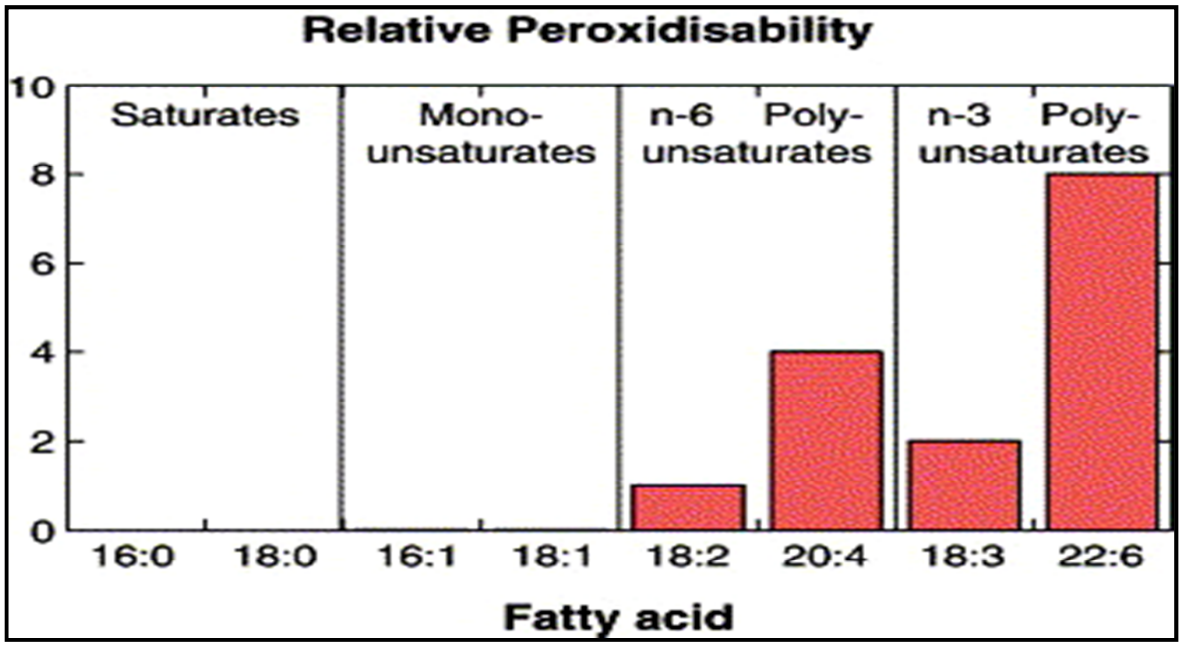

Fig. 6: Relative peroxidisability table. Polyunsaturated fatty acids have the highest sensitivity to oxidative damage and saturated and monounsaturated fatty acids are more resistant (Holman, 1954).

This process, by which membrane fatty acids are oxidatively damaged, is referred to as lipid peroxidation, and is generally thought to be a major mechanism of cell injury in cells under oxidative stress [Catala, 2006]. Lipid peroxidation is the most studied biologically relevant free radical chain reaction and occurs in three stages, initiation, propagation and termination (Fig. 7). A highly reactive radical, such as $HO^{\bullet}$, attacks fatty acids by abstracting one hydrogen from the methyl group on the fatty acyl side chain (bis-allylic hydrogen atom) during the initiation stage. The greater the number of double bonds in a fatty acid side chain, the easier the removal of a hydrogen atom from the chain. The removal of the hydrogen atom leaves behind an unpaired electron on the carbon atom to which it was originally attached, generating a carbon-centered lipid radical which can attack membrane proteins. However, it is most likely to react with O_2 and form a lipid peroxyl radical. The formed lipid peroxyl radical can also attack membrane proteins but are most commonly capable of abstracting a hydrogen atom from an adjacent fatty acyl side chain and therefore propagating the process of lipid peroxidation. One initiating reaction can result in the conversion of several PUFA molecules to lipid peroxides. The length of the propagation chain will depend on factors such as the lipid-protein ratio in a membrane (the chance of a radical reacting with a membrane protein increases as the protein content of the membrane increases), fatty acid composition (the higher the unsaturation of the membrane the longer the propagation chain reaction), O_2 content (the higher the O_2 the higher the chances of O_2 reacting with the lipid radical to yield the lipid peroxyl radical) and the presence or absence of antioxidants within the mitochondria. Termination of the process will come

as a result of all radicals reacting with each other forming non-radical products [Halliwell & Chirico, 1993].

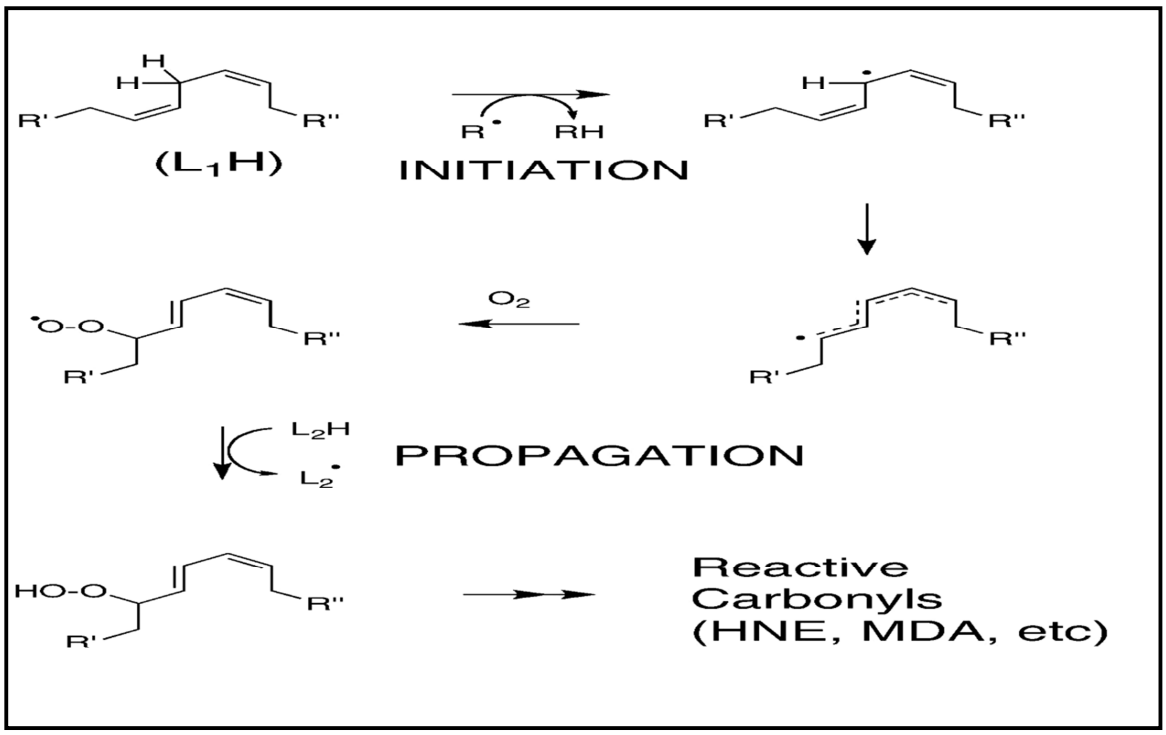

Fig. 7: Lipid peroxidation: Initiation, propagation and reactive metabolites. Abbreviations: unsaturated lipid (L_1H, L_2H); reactive specie ($R^{\bullet}$); oxidized radical (RH); lipid peroxide ($L_2^{\bullet}$); 4-hydroxy-2-nonenal (HNE).

The products of membrane peroxidation are many and include the hydroxyl radical, lipid peroxyl radicals, lipid hydroperoxides as well as hydrocarbons and aldehydes (Fig. 8). It is proposed that much of the cellular and subcellular damage associated with oxidative stress is attributable to the deleterious actions of these peroxidation products [Dotan *et al*., 2004]. Ethane and pentane are the two main volatile hydrocarbons

produced. Ethane results from peroxidation of omega-3 (n-3) PUFAs and pentane result from peroxidation of n-6 PUFAs. Measurements of the exhalation rate of these hydrocarbons enable assessment of the rate of lipid peroxidation *in vivo* [Kneepkens *et al*., 1994]. The aldehydes produced include 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (HHE) and malondialdehyde (MDA). Compared to free radicals, the aldehydes are relatively stable, move between cellular compartments and are capable of attacking targets far from sites of production [Uchida, 2003; Sano & Fukuda, 2008]. Malondialdehyde is in many instances the most abundant individual aldehyde resulting from lipid peroxidation and is a widely used end product to assess oxidative stress [Dotan *et al*., 2004].

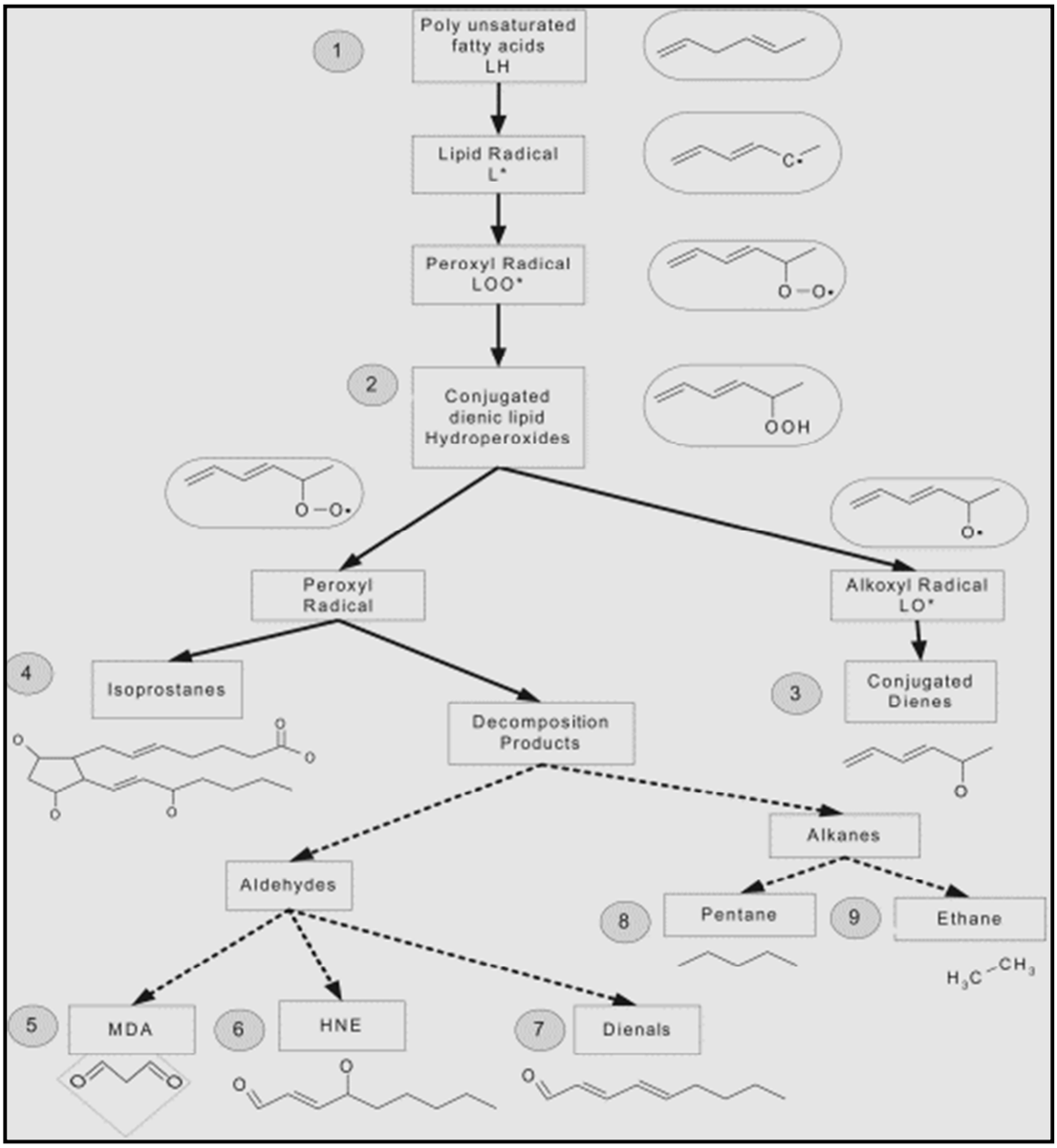

Fig. 8: The products and pathways relating to lipid peroxidation. (1) Polyunsaturated fatty acids. (2) Lipid hydroperoxides. (3) Conjugated dienes. (4) Isoprostanes. (5) Malondialdehyde (MDA). (6) 4-hydroxy-2-nonenal (HNE) (7) Dienals. (8, 9) Alkanes [Dotan *et al*., 2004].

1.4.2. Cell defence mechanism against oxidative stress

1.4.2.1. Systems preventing free radical formation

The antioxidant defence system includes the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzymes. These are endogenous antioxidants that have been well studied.

Superoxide dismutase (SOD): These enzymes catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 (Equation 1). The enzymes are classified into the different groups, iron SOD (Fe-SOD), manganese SOD (Mn-SOD), copper-zinc SOD (Cu-Zn-SOD) and nickel SOD (Ni-SOD) on the basis of the metal at the active sites [Culotta *et al*., 2006). The transition metals appear to function in the dismutation reaction by undergoing alternate oxidation and reduction [Halliwell & Gutteridge, 2007].

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 \text{ (SOD)} \qquad \text{(Equation 1)}$$

Catalase (CAT): The end product of dismutation reaction, H_2O_2, can be removed by the action of the enzyme CAT which can remove H_2O_2 present in high concentrations. Catalase directly decomposes H_2O_2 to H_2O and O_2 (Equation 2).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (CAT)} \qquad \text{(Equation 2)}$$

Glutathione peroxidise (GPx): Glutathione peroxidase is a peroxidase enzyme, whose electron donor is glutathione (GSH). GSH is oxidized to oxidized glutathione (GSSG)

through disulfide bridge formation (Equation 3). Glutathione peroxidase has a high affinity for H_2O_2 and can remove it even when present in low concentration [Li *et al*., 2009].

-ROOH + 2GSH → -ROH + GSSG (GPx) (Equation 3)

1.4.2.2. Systems eliminating formed ROS

The most extensively studied endogenous non-enzymatic antioxidants include glutathione (GSH), NADPH and trehalose.

Glutathione: GSH is a thiol-containing tripeptide (L-glutamyl-L-cystinylglycine) and a major antioxidant molecule of the cell playing a vital role in buffering the cell against ROS [Stephen & Jamieson, 1996]. GSH is synthesized from the reaction between glutamate and cysteine through the action of two distinct enzymes, glutamylcysteine synthase and glutathione synthase. Glutathione can directly reduce a number of oxidants, through the enzyme glutathione peroxidase (GPx), giving rise to its oxidized form GSSG in which two GSH molecules join via the oxidation of the –SH groups of the cysteine residue to form a disulphide bridge. Glutathione acts as a cofactor for the enzyme GPx, thus serving as an indirect antioxidant donating the electrons necessary for the reduction of oxidants [Gul *et al*., 2000].

NADPH: is an important reducing equivalent in cellular metabolism and it has been shown to also function as an antioxidant [Minard & McAlister-Henn, 2001]. NADPH is

thought to provide reducing power for GSH in an NADPH-dependent reaction catalyzed by glutathione reductase (GR).

Trehalose: This disaccharide is widely distributed and has been shown to function as a radical scavenger, protecting intracellular constituents from oxidative damage and heat shock. Trehalose was shown to offer little protection to antioxidant enzymes SOD and CAT against oxidative damage and did not confer oxidative stress resistance to *Saccharomyces cerevisiae*, however it is able to directly eliminate H_2O_2 and $O_2^{\bullet-}$ in certain cells in a dose dependent manner [Benaroudi *et al*., 2001; Luo *et al*., 2008; Mahmud *et al*., 2010].

1.4.3. ROS-Induced cell death

Apoptosis is a form of programmed cell death with an important role in development and homeostasis of multicellular organisms [Madeo *et al*., 1997]. Programmed cell death is a ubiquitous process in metazoan organisms. Apoptosis allows the rapid removal of unwanted or damaged cells that could cause damage to the surrounding cells with their cytoplasmic contents. The pathways by which cells in multicellular organisms trigger cell death have been well characterized and since the discovery of apoptosis in yeasts [Madeo *et al*., 1997], many yeast proteins involved in apoptosis, which are orthologs of mammalian apoptosis proteins, have been studied [Camona-Guitierrez *et al*., 2010] . A cell triggered to undergo apoptosis activates a widely described cascade of molecular events, which results in programmed cell death. A distinctive scenario for programmed cell death development includes activation of the mitogen-activated proteins (MAP)

kinase cascade, accumulation of ROS, release of cytochrome c from mitochondria into cytoplasm as a consequence of opening the permeability transition pore in the mitochondrial membrane and activation of caspases [Skulachev, 1996]. Reactive oxygen species production is identified as a key cellular event common to the known scenarios of apoptosis in yeast and animal cells [Madeo *et al*., 1999; Carmona-Gutierrez *et al*., 2010]. A set of phenotypic alterations, such as externalization of phosphatidylserine (PS), chromatin condensation and DNA fragmentation are characteristic of mammalian and yeast apoptosis [Madeo *et al*., 1997; Carmona-Gutierrez *et al*., 2010].

1.4.4. Influence of exogenous fatty acids on oxidative stress

Exogenous fatty acids are fatty acids not synthesized in the host but are taken in as part of the diet or supplemented in the growth medium [Martin *et al*., 2007]. When supplemented in the medium, they are imported into the cell through various mechanisms such as the transmembrane lipid translocation (flip flop mechanisms) [Gurtovenko & Vattulainen, 2007]. They are then significantly incorporated into membrane phospholipids. Phospholipid fatty acid composition is a major determinant of membrane (including mitochondrial membrane) function and alteration of the membrane will have a major impact on cellular function. For example, the function of proteins that regulate membrane flux across the mitochondrial membrane depends on their position and conformation, which is significantly influenced by membrane phospholipid composition [Rohrbach, 2009; Landolfo *et al*., 2010].

Linoleic acid (C18:2 n-6), a PUFA, showed toxicity to the yeast *S. cerevisiae* when the fatty acid was supplemented into the growth medium. Linoleic acid (C18:2 n-6) was readily incorporated into the yeast mitochondrial membrane influencing the membrane fatty acid content and composition [Ferreira *et al.*, 2011]. This resulted in an increase in the unsaturation index and increased levels of lipid peroxidation due to oxidative stress. Furthermore, these authors reported that exposure of the yeast to C18:2 n-6 significantly increased the levels of the antioxidant enzymes, glutathione (GSH) and catalase (CAT) compared to the control, suggesting enzymatic reactions of GSH synthesis and defence against H_2O_2 are increased during exposure to C18:2 n-6.

In another study by Cortes-Rojo and co-workers (2009), supplementation of linolenic acid (C18:3 n-3) to isolated mitochondria of *S. cerevisiae* resulted in increased fatty acid content of the yeast mitochondria. Furthermore, sensitivity of fatty acids to peroxidation was higher due to the higher number of double bonds in the fatty acids. Interestingly, supplementation of isotope-reinforced polyunsaturated fatty acids protected the yeast *S. cerevisiae* from lipid peroxidation since these fatty acids were resistant to the abstraction of the hydrogen atom from the double bond [Hill *et al*., 2011].

Interestingly, when *S. cerevisiae* is cultivated in high-sugar-containing media, without exogenous fatty acids, it experiences oxidative stress, due to the release of intermediates of lipid biosynthesis [Landolfo *et al*., 2010]. This increase in reactive intermediates can be counteracted by the incorporation of exogenous, monounsaturated, oleic acid (C18:1 n-9), thus mitigating oxidative stress and damage under unfavourable conditions.

1.5. Purpose of study

With the above as background, it became the aim of this study to:

1. Screen saturated, monounsaturated and polyunsaturated fatty acids and evaluate the effect they have on biofilm formation of *C. albicans* and *C. dubliniensis* (Chapter 2).
2. Evaluate the effect of the antifungal fatty acids (selected from Chapter 2) on mitochondria, oxidative stress and apoptosis of *C. albicans* and *C. dubliniensis* (Chapter 3).
3. Evaluate the synergistic effect of the antifungal compound, Amphotericin B (AmB), and an antifungal fatty acid on *C. albicans* and *C. dubliniensis* biofilms (Chapter 4).

1.6. References

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Chapter 2

Effect of fatty acids on biofilm formation of *C. albicans* and *C. dubliniensis*

Parts of the chapter have been published in the journal, Marine Drugs 8 (10), 2579-2604, (2010).

2.1. Summary

The effect of saturated, monounsaturated and polyunsaturated fatty acids (PUFAs) on biofilm formation by the human pathogens *Candida albicans* and *Candida dubliniensis* was investigated. Several fatty acids inhibited reduction of XTT (used for the determination of metabolic activity) of biofilms of both species, with *C. albicans* being the more susceptible. Three PUFAs (stearidonic acid, eicosapentaenoic acid and docosapentaenoic acid) reduced metabolic activity and biofilm biomass production of both *C. albicans* and *C. dubliniensis*. Ultrastructural changes were observed between supplemented and unsupplemented biofilms. Biofilms of both species supplemented with the three inhibitory fatty acids showed formation of rough cell walls and fibrillar structures in *C. dubliniensis* which may be due to increased oxidative stress. However, biofilms supplemented with linoleic acid, which was moderately inhibitory towards metabolic activity and biomass production, showed a smooth cell surface similar to the control. These results indicate that some PUFAs may be useful in the supplementation and/or prevention of biofilms formed by these pathogenic yeasts.

2.2. Introduction

Candida albicans and *Candida dubliniensis* are dimorphic yeasts, able to grow both as yeasts and mycelia [Molero *et al*., 1998; Ramage *et al*., 2001a]. Several members of the genus *Candida* exist as commensals of the human gastrointestinal and genitourinary tract in healthy individuals [Berman & Sudbery, 2002]. However, in individuals whose immune system is compromised, such as those that are HIV positive, *C. albicans* can cause diseases ranging from superficial infections to deep seated mycoses [Neofytos *et al*., 2010]. *Candida dubliniensis* is a species closely related to *C. albicans* and a causative agent of oropharyngeal candidiasis in immunocompromised humans [Coleman *et al*., 1997; Jabra-Rizk *et al*., 2000].

Fatty acids have been known to have antibacterial and antifungal properties. Capric acid (C10:0) and lauric acid (C12:0) are known to have anti-*Candida* effects, inhibiting growth of planktonic cells [Bergusson *et al*., 2001], and butyric acid (C4:0) was shown to inhibit hyphal formation by *C. albicans* [Noverr & Huffnagle, 2004]. Fatty acids vary in length and saturation, with naturally occurring fatty acids having a chain length of 4 to 28 chains which may be saturated or unsaturated [Sylvain *et al*., 2009]. Hydrophobic groups of saturated fatty acids play an important role in bioactivity [Branen *et al*., 1980]. In general, the antifungal efficiency of fatty acids increases with an increase in chain length. However, the increase of hydrophobicity with longer chain length may reduce their solubility in aqueous solutions [Ouattara *et al*., 1997] and hydrophobic groups may be prevented from reaching sufficient concentrations to interact with acyl chains of membrane phospholipids [Wang & Johnson, 1992].

Biofilm formation is a major virulence factor in the pathogenicity of *Candida* species, partly due to their increased resistance to antifungal supplementation [Coleman *et al*., 1998; Ballie & Douglas, 1999]. Since biofilm associated infections have many clinical and economic consequences, recent research into the pathogenicity of *Candida* species has focused on the prevention and management of these biofilms. Although fatty acids have shown promise against planktonic *Candida* cells, no information is available regarding the effect of fatty acids against more resistant biofilms. Therefore, the aim of this study was to determine the effect of medium to very long chain fatty acids on biofilm formation by *C. albicans* and *C. dubliniensis*.

2.3. Materials and Methods

2.3.1. Strains used

Candida albicans CBS 562T [clinical strain, interdigital mycoses (Uruguay)] and *Candida dubliniensis* NRRL Y-17841T [clinical strain, oral cavity of HIV infected patient (Ireland)] were used in this study and were maintained on yeast malt extract (YM) agar plates (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. The strains were also stored on agar slants at 4 ℃.

2.3.2. Biofilm formation

Cells of *C. albicans* and *C. dubliniensis* were grown separately on YM agar plates and incubated at 30 ℃ for 24 hours. After incubation, a loop-full of the cells was inoculated into 20 mL of yeast nitrogen base (YNB) glucose medium (10 g/L glucose, 6.7 g/L YNB)

and incubated at 30 ℃ for 48 hours. Cells were was hed twice with sterile phosphate buffered saline (PBS) by centrifugation for 5 minutes at 4000*g* with a Heraeus® Megafuge® 1.0R centrifuge (SepSci, SA) and diluted in filter sterilized RPMI-1640 medium (Sigma-Aldrich, UK) to a standardized cell concentration of 1 x 10^6 cells/mL. Biofilms were allowed to form by first incubating the cells at 37 ℃ for 1 hour, to allow adherence of cells to the surface [Samaranayake *et al*., 1995]. Non-adherent cells were removed by washing twice with PBS.

2.3.3. Measurement of cellular viability (XTT assay)

Mature biofilms were formed in 96-well microtiter plates (Corning Incorporated, Costar®, USA) in the presence and absence (control) of 1 mM fatty acids (Table 1) diluted in filter sterilized RPMI-1640. Different fatty acids are used because the antimicrobial activity of fatty acids increases with an increase in chain length, however very long fatty acids will have decreased activity due to their increased hydrophobicity and reduced solubility. Fatty acids were used to select antifungal fatty acids more active against biofilms [Sylvain *et al*., 2009]. Briefly, a volume of 100 µL RPMI-1640 medium solution was dispensed and incubated into the 1 hour old biofilm, prepared as described above, at 37 ℃ for an additional 47 hours. After incubation, we lls were washed twice with PBS to remove non-adherent cells. The reduction of (2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (XTT) (Sigma Aldrich, USA) in the presence of metabolic activity was used to examine the yeast viability, according to the method of Ramage and co-workers (2001b). The XTT solution was prepared with 1 %

XTT w/v and 1 mM menadione. XTT is converted to the diffusible, water soluble formazan that is colored and is easily measured in cellular supernatants in terms of optical density at a wavelength of 492 nm. The experiment was done in triplicate, with a sample number (n) of eight and the average and standard deviations were calculated.

Table 1: Saturated, monounsaturated and polyunsaturated fatty acids, used for the reduction of XTT.

Fatty Acids Common Name	IUPAC Name	Abbreviations
Saturated fatty acids [a]		
Lauric acid	Dodecanoic acid	C12:0
Stearic acid	Octadecanoic acid	C18:0
Arachidic acid	Eicosanoic acid	C20:0
Lignoceric acid	Tetracosanoic acid	C24:0
Monounsaturated fatty acids [b]		
Palmitoleic acid	*cis*-9-Hexadecenoic acid	C16:1 n-7
Oleic acid	*cis*-9-Octadecenoic acid	C18:1 n-9
Petroselinic acid	*cis*-6-Octadecenoic acid	C18:1 n-12
Eicosenoic acid	*cis*-13-Eicosenoic acid	C20:1 n-7
Erucic acid	*cis*-13-Docenoic acid	C22:1 n-9
Nervonic acid	*cis*-15-Tetracosenoic acid	C24:1 n-9

Fatty Acids Common Name	IUPAC Name	Abbreviations
Polyunsaturated fatty acids [c]		
Palmitolinoleic acid	*cis*-9,12-Hecadecadienoic acid	C16:2 n-4
Hexadecatrienoic acid	*cis*-7,10,13-Hexadecatrienoic acid	C16:3 n-3
Linoleic acid	*cis*-9,12-Octadecadienoic acid	C18:2 n-6
α-Linolenic acid	*cis*-9,12,15-Octadecatrienoic acid	C18:3 n-3
γ-Linolenic acid	*cis*-6,9,12-Octadecatrienoic acid	C18:3 n-6
Stearidonic acid	*cis*-6,9,12,15-Octadecatetraenoic acid	C18:4 n-3
Eicosadienoic acid	*cis*-11,14-Eicosadienoic acid	C20:2 n-6
Eicosatrienoic acid	*cis*-11,14,17-Eicosatrienoic acid sodium salt	C20:3 n-6
ω-Arachidonic acid	*cis*-8,11,14,17-Eicosatetraenoic acid	C20:4 n-3
Arachidonic acid	*cis*-5,8,11,14-Eicosatetraenoic acid	C20:4 n-6
Eicosapentaenoic acid	*cis*-5,8,11,14,17-Eicosapentaenoic acid	C20:5 n-3
Docosatrienoic acid	*cis*-13,16,19-Docosatrienoic acid	C22:3 n-6

Fatty Acids Common Name	IUPAC Name	Abbreviations
Polyunsaturated fatty acids [c]		
Docosatetraenoic acid	*cis*-10,13,16,19-Docosatetraenoic acid	C22:4 n-6
Docosapentaenoic acid	*cis*-7,10,13,16,19-Docosapentaenoic acid	C22:5 n-3
Docosahexaenoic acid	*cis*-4,7,10,13,16,19,Docosahexaenoic acid	C22:6 n-3
Tetracosatetraenoic acid	*cis*-9,12,15,18-Tetracosatetraenoic acid	C24:4 n-6
Tetracosapentaenoic acid	*cis*-9,12,15,18,21-Tetracosapentaenoic acid	C24:5 n-3
Tetracosapentaenoic acid	*cis*-6,9,12,15,18-Tetracosapentaenoic acid	C24:5 n-6
Tetracosahexaenoic acid	*cis*-6,9,12,15,18,21-Tetracosahexaenoic acid	C24:6 n-3

[a]-Saturated fatty acids are straight chains and consists of a carbon chain with single bonds.

[b]-Monounsaturated fatty acids contain one double carbon-carbon bond in the carbon chain.

[c]-Polyunsaturated fatty acids contain more than one double carbon-carbon bond in the carbon chain.

2.3.4. Biofilm biomass determination

Mature biofilms were formed in sterile Petri dishes (Lasec, SA) in the presence and absence (control) of 1 mM fatty acid (Table 2) diluted in filter sterilized RPMI-1640 medium. These free fatty acids were selected following the XTT assay. Twenty milliliters of the RPMI-1640 medium solution were added to the 1 hour old biofilms, prepared as described above and mature biofilm formed by incubating at 37 ℃ for an additional 47 hours. After incubation, biofilms were washed twice with PBS to remove non-adherent cells, scraped off and resuspended in PBS. Cells were filtered on pre-weighed 0.2 µm cellulose acetate filters (Lasec, SA) and dried to constant dry weight in an oven at 37 ℃ for 48 hours, after which the biomass was determined. The experiment was done in duplicate and the average and range calculated.

Table 2: Polyunsaturated free fatty acids selected for biofilm biomass determination.

Fatty Acids Common Name	Abbreviations
Oleic acid	C18:1 n-9
Linoleic acid	C18:2 n-6
α-Linolenic acid	C18:3 n-3
γ-Linolenic acid	C18:3 n-6
Stearidonic acid	C18:4 n-3
Eicosenoic acid	C20:1 n-7
Eicosadienoic acid	C20:2 n-6
Arachidonic acid	C20:4 n-6
Eicosapentaenoic acid	C20:5 n-3
Docosapentaenoic acid	C22:5 n-3

2.3.5. Morphological examination (ultrastructure)

Antifungal fatty acids (C18:4 n-3, C20:5 n-3 and C22:5 n-3) and a non-antifungal fatty acid (C18:2 n-6) (positive control) were chosen following biomass determination to evaluate their effect on the morphology of the biofilms. Mature biofilms were formed in chamber slides (Lab-Tek® Chamber Slide™ System, USA) containing silicone rubber disks (diameter 5.5 mm) in the presence and absence (control) of 1 mM fatty acids diluted in RPMI-1640 medium. Briefly, RPMI-1640 medium solution was added to the 1 hour old biofilms, prepared as described above and incubated at 37 ℃ for an additional

47 hours. After incubation, the silicone rubber disks were removed and fixed for 2 hours using 3% (v/v; 1.0 M) sodium phosphate buffered glutaraldehyde, followed by fixation for 1 hour with a similarly buffered solution of osmium tetroxide (1% m/v). The disks were dehydrated in a graded series of ethanol solutions (50%, 70% and 95%) for 20 minutes and absolute ethanol for 1 hour. They were then critical-point dried, mounted and coated with gold to make them electrically conductive and finally visualized on a Shimadzu SSX550 SEM (Japan) microscope according to the method of van Wyk and Wingfield (1991).

2.4. Results and Discussion

2.4.1. Measurement of cellular viability (XTT assay)

Figure 1 indicates the effect of fatty acids on XTT reduction (an indicator of metabolic activity) by biofilms of *C. albicans* and *C. dubliniensis*. Although certain fatty acids were able to increase the reduction of XTT especially in the case of *C. dubliniensis*, several PUFAs inhibited metabolic activity of both species with *C. albicans* being more susceptible.

Lauric acid (C12:0) was the most effective saturated fatty acids, inhibiting about 64% biofilm metabolic activity of *C. albicans*. A similar inhibitory effect against *C. albicans* was observed by Bergsson and co-workers (2001) who reported that C12:0 inhibited growth of planktonic cells due to disorganization and shrinking of the cytoplasm because of disrupted or disintegrated plasma membranes.

Several polyunsaturated fatty acids (C16:2 n-4, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-3 and C20:4 n-6) were effective at inhibiting mitochondrial metabolic activity of *C. albicans* between 40% and 84%. Four polyunsaturated fatty acids (C16:3 n-4, C18:4 n-3, C20:5 n-3 and C22:5 n-3) were effective against both *C. albicans* and *C. dubliniensis* and inhibited mitochondrial metabolism by between 53% and 97%. Other fatty acids did not significantly inhibit mitochondrial metabolism of either *C. albicans* or *C. dubliniensis* biofilms and as a result were not used in further experiments.

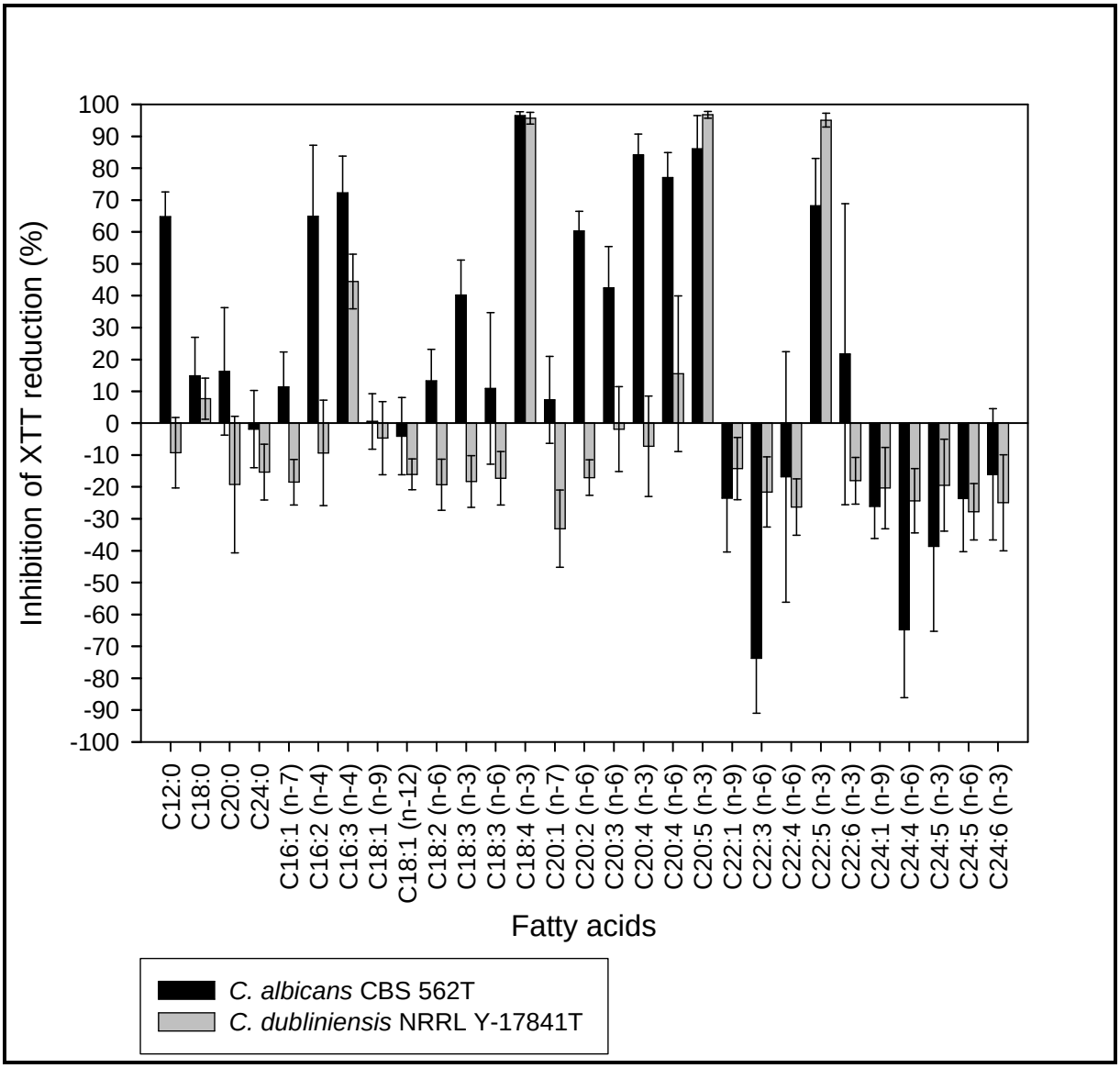

Fig. 1: Effect of saturated, monounsaturated and polyunsaturated fatty acids on the metabolic activity of biofilms by *C. albicans* and *C. dubliniensis.* The positive side (upper) is an indicator of reduced reduction of XTT, an indicator of mitochondrial metabolic activity, and the negative side (lower) is an indicator of increased reduction of XTT. The percentage inhibition values were determined compared to unsupplemented controls. Values are the averages of eight samples and standard deviations are indicated by error bars.

2.4.2. Biofilm biomass determination

Although several authors use the XTT reduction assay as an indicator of biofilm biomass, Kuhn and co-workers (2003) has cautioned against this approach. Therefore, the effect of the PUFAs on biofilm biomass production was determined by dry weight. As indicated in figure 2, biofilm biomass production by both *C. albicans* and *C. dubliniensis* were susceptible to supplementation by C18:1 n-9, C18:4 n-3, C20:1 n-7, C20:2 n-6, C20:4 n-6, C20:5 n-3 and C22:5 n-3. These PUFAs resulted in a reduction of biofilm biomass of more than 40% against both species. However, C18:2 n-6, C18:3 n-3 and C18:3 n-6 reduced biofilm biomass by less than 40%. Only three PUFAs C18:4 n-3, C20:5 n-3 and C22:5 n-3 showed a linear inhibitory correlation between metabolic activity and biomass production for both species, while C18:3 n-3, C20:2 n-6 and C20:4 n-6 showed a linear inhibitory correlation between metabolic activity and biomass determination only against *C. albicans*. These results may indicate the ability of the biofilms to obtain energy through pathways that do not require mitochondrial metabolism.

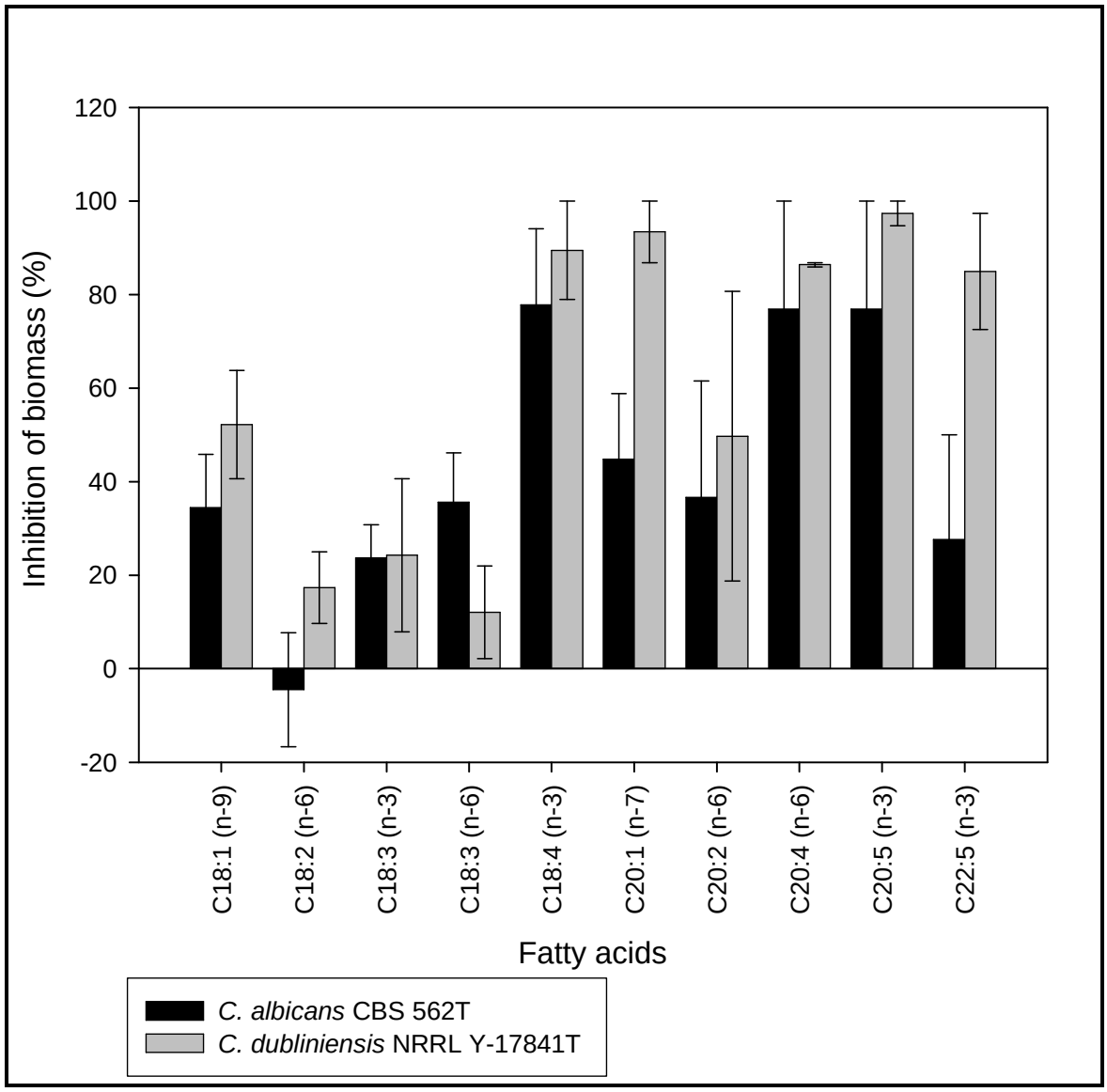

Fig. 2: The percentage inhibition of biofilm biomass of *C. albicans* and *C. dubliniensis* compared to unsupplemented controls. Biofilms were grown in the presence of 1mM monounsaturated and polyunsaturated fatty acids and biofilm dry weight was determined on pre-weighed filters. Values are the average of two experiments with the range is indicated by error bars.

2.4.3. Morphological examination (ultrastructure)

Figure 3 depicts biofilms of *C. albicans* grown in the presence and absence of 1 mM fatty acids (C18:2 n-6, C18:4 n-3, C20:5 n-3 and C22:5 n-3). In the absence of the PUFAs, the cell surface appeared smooth (Fig. 3A) and when grown in the presence of inhibitory PUFAs, cells had a rough appearance with protuberances all around (Fig. 3C, D & E). Interestingly, figure 3B shows cells grown in the presence of C18:2 n-6 and the cell surface appeared smooth, similar to the control. Similar results were also observed for biofilms of *C. dubliniensis* when grown in the absence (Fig. 4A), and the presence (Fig. 4C, D & E), of the PUFAs with protuberances and fibrillar structures visible on the cell surface. Furthermore, the cell surface of cells supplemented with C18:2 n-6 (Fig. 4B) appeared smooth as it was with the control. Similar rough cell surfaces were observed when *C. albicans* was exposed to miconazole [Nollin & Borgers, 1975], which is known to cause an increase in reactive oxygen species in *Candida* biofilm cells [Vandenbosch *et al*., 2010]. Lemar and co-workers (2005) also found that *C. albicans* cells were not smooth in the presence of alyl alcohol, which increases oxidative stress. Furthermore, in a study by Leeuw and co-workers (2010) on the yeast *Cryptococcus curvatus* grown on oxidized lipids, protuberances were observed on the cell surface. We therefore speculate that the changes in cell surface in the presence of PUFAs might be due to increased lipid peroxidation and resultant oxidative stress. This will be evaluated in the next chapter.

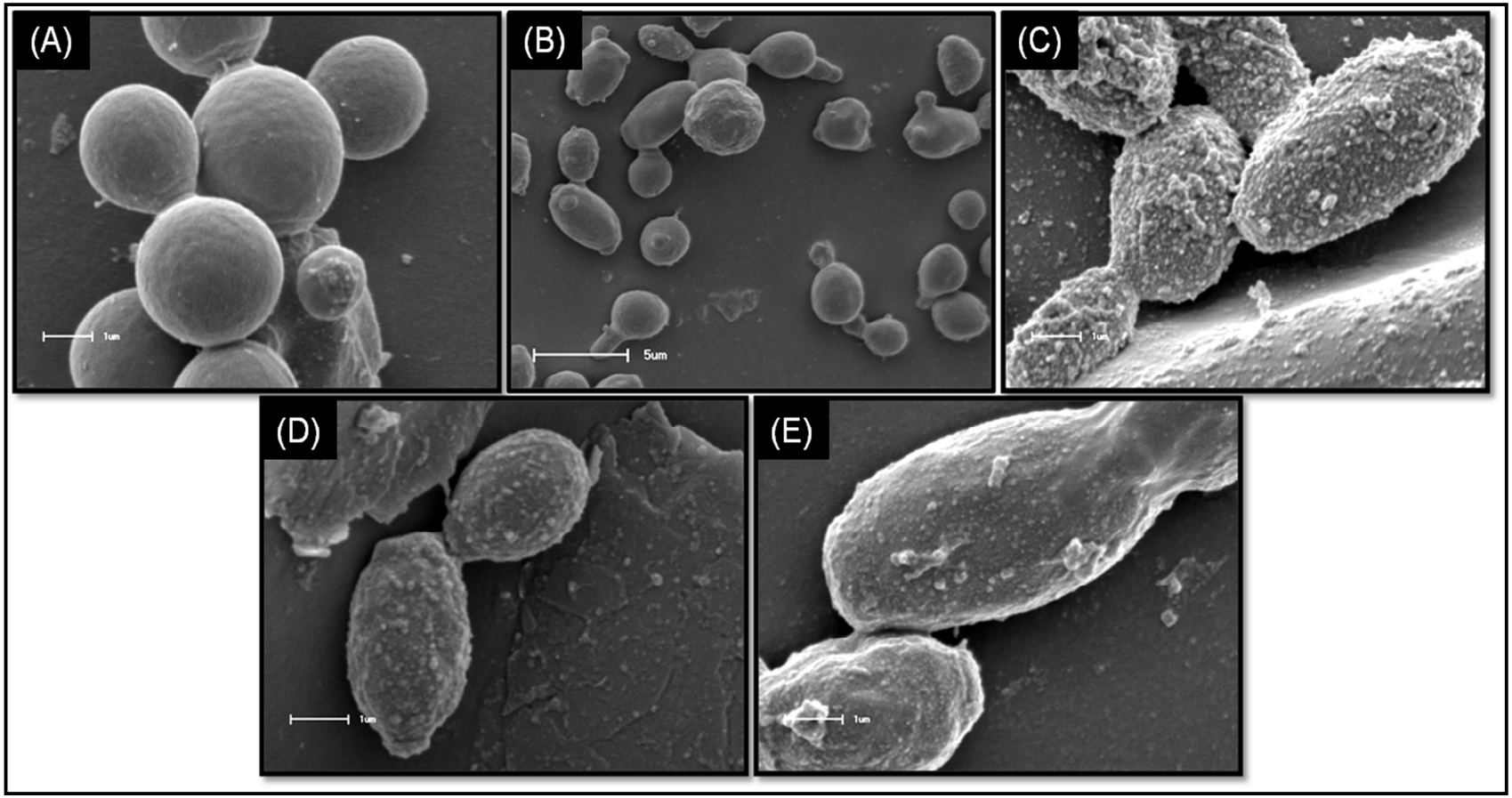

Fig. 3: Scanning electron micrograph showing cells of *C. albicans*, control biofilms **(A)** and biofilms supplemented with 1 mM C18:2 n-6 **(B)**, C18:4 n-3 **(C)**, C20:5 n-3 **(D)** and C22:5 n-3 **(E)**.

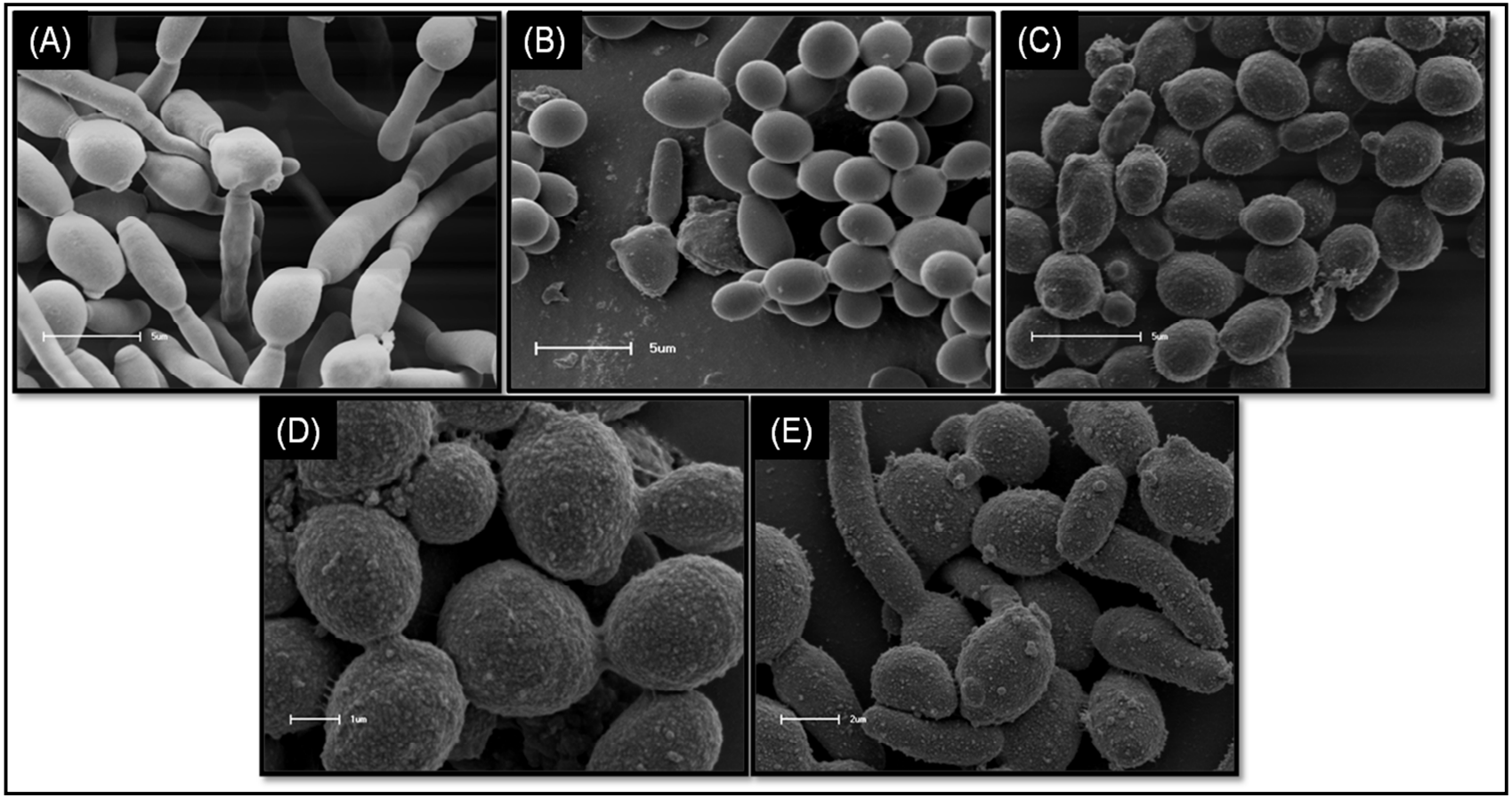

Fig. 4: Scanning electron micrograph showing cells of *C. dubliniensis*, control biofilms **(A)** and biofilms supplemented with 1 mM C18:2 n-6 **(B)**, C18:4 n-3 **(C)**, C20:5 n-3 **(D)** and 22:5 n-3 **(E)**.

2.5. Conclusions

This study showed that several polyunsaturated fatty acids have antifungal properties against biofilm formation of *C. albicans* and *C. dubliniensis*, with *C. albicans* being the most susceptible. Both species were resistant to supplementation by saturated and monounsaturated fatty acids. Lauric acid (C12:0), was the only exception and significantly reduced the reduction of XTT by biofilms of *C. albicans*. Polyunsaturated fatty acids, especially C18:4 n-3, C20:5 n-3 and C22:5 n-3 were able to inhibit biofilm formation by reducing reduction of the XTT salt and biomass production of both *C. albicans* and *C. dubliniensis*. These PUFAs also affected cellular morphology of biofilms of both *C. albicans* and *C. dubliniensis* resulting in formation of cell wall protuberances. This we proposed might be due to increased oxidative stress when these PUFAs are incorporated into the mitochondrial membrane phospholipids. This hypothesis will be further investigated in the next chapter.

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Chapter 3

Effect of fatty acids on mitochondria, oxidative stress and apoptosis in *C. albicans* and *C. dubliniensis*

3.1. Summary

Polyunsaturated fatty acids (PUFAs) have been known to have antifungal properties but the mode by which they induce their action is not always clear. The aim of the study was to investigate increased oxidative stress as possible mode of action of the antifungal PUFAs (stearidonic acid, eicosapentaenoic acid and docosapentaenoic acid) which we have identified as inhibitory towards biofilm formation of *C. albicans* and *C. dubliniensis*. When biofilms of *C. albicans* and *C. dubliniensis* were exposed to the PUFAs there was an increase in accumulation of intracellular reactive oxygen species (ROS), which increased lipid peroxidation and eventually lead to increased oxidative stress. Increase in ROS-induced oxidative stress resulted in reduced mitochondrial membrane potential, which is a major factor in the induction of apoptosis. The externalization of phosphatidylserine (PS), an indicator of early stages of apoptosis, was examined using the Annexin V-FITC assay. In addition, DNA fragmentation and nuclear condensation and fragmentation were analyzed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and 4', 6-diamidino-2-phenylindole (DAPI) staining for late stages of apoptosis. We found that polyunsaturated fatty acids were incorporated into membrane phospholipids, increasing oxidative stress and eventually resulting in cell death of both *C. albicans* and *C. dubliniensis* due to the induction of apoptosis.

3.2. Introduction

Apoptosis is a highly regulated form of programmed cell death, involved in rapid removal of unwanted or damaged cells, which is important for development and homeostasis of metazoan organism as well as unicellular organisms such as yeasts [Madeo *et al.*, 1997]. Apoptosis is characterized by a set of distinct morphological markers and yeast cells show typical apoptotic markers such as the externalization of phosphatidylserine, DNA fragmentation as well as chromatin fragmentation and condensation. Furthermore, the cytochrome c release from the mitochondria [Ludovico *et al.*, 2002], reduced mitochondrial membrane potential [Pozniakovsky *et al.*, 2005] and formation of membrane enclosed cell fragments, called apoptotic bodies, all mark programmed death of yeast cells [Wissing *et al.*, 2004].

Madeo and co-workers (1997) first discovered the apoptotic pathway similar to those in multicellular organism, in the yeast *Saccharomyces cerevisiae*. Since then several endogenous and exogenous stimuli have been shown to be responsible for the induction of apoptosis in this yeast. Some of these stimuli include mutation or deletion of specific genes [Madeo *et al.*, 1997] and exogenous stimuli such as acetic acid and hydrogen peroxide [Madeo *et al*, 1999; Ludovico *et al*, 2001; Ribeiro *et al*, 2006]. *Saccharomyces cerevisiae* is an ideal model for the investigation of the oxidative toxicity of diverse compounds, because it is genetically well defined, easy to manipulate and its oxidative defense system is well characterized [Ferreira *et al*., 2011].

Research has also shown the existence of apoptotic pathways in the human pathogen *Candida albicans*. In this yeast, antimicrobial peptides such as melittin [Park & Lee, 2010], psacotheasin [Hwang *et al*., 2011a], pleurocidin [Cho & Lee, 2011] and papiliocin

[Hwang *et al*., 2011b] were found to be antifungal through the induction of apoptosis. These peptides were reported to induce oxidative stress through increased production of reactive oxygen species (ROS) and lipid peroxidation of mitochondrial membranes. Accumulation of ROS has long been proposed to be a key event in the apoptotic pathway [Perrone *et al*., 2008].

The toxicity of fatty acids is dose dependent and relates to chain length and unsaturation, with higher concentrations of fatty acids, especially polyunsaturated fatty acids (PUFAs), causing cell death through apoptosis [Lima *et al*., 2002]. In the recent work by Ferreira and co-workers (2011), they reported that supplementation of media with PUFAs induced the apoptotic pathway in *S. cerevisiae*.

The aim of the study was therefore to investigate whether the observed antifungal activity of PUFAs (Chapter 2) can be through the induction of the oxidative stress, leading to apoptosis in *C. albicans* and *C. dubliniensis* biofilms.

3.3. Materials and Methods

3.3.1. Strains used

Candida albicans CBS 562T and *Candida dubliniensis* NRRL Y-17841T were used in this study and were maintained on yeast malt extract (YM) agar plates (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. The strains were also stored on agar slants at 4 ℃.

3.3.2. Biofilm formation

Cells of *C. albicans* and *C. dubliniensis* were grown separately on YM agar plates and incubated at 30 ℃ for 24 hours. After incubation, a loop-full of the cells was inoculated into 20 mL of yeast nitrogen base (YNB) glucose medium (10 g/L glucose, 6.7 g/L YNB) and incubated at 30 ℃ for 48 hours. Cells were washed twice with sterile phosphate buffered saline (PBS) by centrifugation for 5 minutes at 4000*g* with a Heraeus® Megafuge® 1.0R Centrifuge and diluted in filter sterilized RPMI-1640 medium (Sigma Aldrich, USA) to a standardized cell concentration of 1 x 10^6 cells/mL. Biofilms were allowed to form by first incubating the cells at 37 ℃ for 1 hour, to allow adherence of cells to the surface [Samaranayake *et al*., 1995]. Non-adherent cells were removed by washing twice with sterile PBS. Different PUFAs (C18:2 n-6, C18:4 n-3, C20:5 n-3 and C22:5 n-3) with a final concentration of 1 mM were added after 1 hour incubation and mature biofilms were formed at 37 ℃ for an additional 47 hours. Ethanol was used as a control.

3.3.3. Phospholipid fatty acid analyses

Biofilms were washed twice with sterile PBS, scraped off using a cell scraper and resuspended in sterile PBS. Total lipids of the biofilms were extracted with chloroform:methanol (2:1) [Folch *et al*.,1957]. The total lipids were separated into different fractions using solid phase 0.50 g Si extraction columns (Separations, SA) according to the method of Bossio & 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 N_2 at 32 ℃. Fatty acid methyl esters (FAMEs) of the phospholipid fractions were prepared using methanol-BF_3 [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 ℃ (h old 2 minutes; 4 ℃/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 ℃. Hy drogen, 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 FAMEs 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 samples. The unsaturation index was calculated (Equation 1). This experiment was performed in duplicate.

Unsaturation Index = 1 x [% monoenoic fatty acids] + 2 x [% dienoic fatty acids] + 3 x [% trienoic fatty acids] + 4 x [% tetraenoic fatty acids] + 5 x [% pentaenoic fatty acids] (Equation 1)

3.3.4. Oxidative stress

3.3.4.1. Accumulation of reactive oxygen species (ROS)

Accumulation of intracellular ROS production was measured using the fluorescent dye, 2',7-dichlorofluorescin diacetate (DCFHDA) (Sigma Aldrich, USA). Briefly, biofilms were prepared as described above in black 96-well microtiter plates (Corning Incorporated, Costar®, USA) by incubating plates at 37 ℃ for 24 hours. Biofilms were washed twice with sterile PBS to remove non-adherent cells and 100 µL of filter sterilized RPMI-1640 medium containing 1mM of the PUFAs (C18:2 n-6, C18:4 n-3, C20:5 n-3 and C22:5 n-3) were added. An ethanol control was included. A volume of 10 µM DCFHDA was simultaneously added with the PUFAs and the plate was incubated at 37 ℃ for an additional 12 hours [Kobayashi *et al*., 2002]. Fluorescence was measured after incubation using a SpectraMax M2 plate reader (Molecular Devices, USA) at an excitation/emission wavelength of 485/535 nm, respectively. The experiment was performed in triplicate.

3.3.4.2. TBARS (lipid peroxidation) assay

Biofilms were prepared in Petri dishes and supplemented with PUFAs as described above. Biofilms were washed twice with sterile PBS and scraped off using a cell scraper and resuspended in sterile PBS. The cells were disrupted with the French press cell disrupter (Constant Cell Disruption Systems) at a pressure of 36 kpsi. The formation of malondialdehyde (MDA) was monitored according to the TBARS Assay Kit (Cayman chemicals, USA). The OD of malondialdehyde-thiobarbituric acid (MDA-TBA) adduct formed by the reaction of MDA and TBA under high temperatures (90-100 ℃) and

acidic conditions was measured using a SpectraMax M2 plate reader at a wavelength between 530-540 nm. The experiment was performed in duplicate.

3.3.5. Apoptotic markers

3.3.5.1. Mitochondrial membrane potential

The mitochondrial membrane potential of biofilms prepared in black 96-well microtiter plates, and supplemented with PUFAs as described above, was measured. After incubation, wells were washed twice with sterile PBS and the plates assayed according to the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman chemicals, USA) using a SpectraMax M2 plate reader. The lipophilic cationic dye, 5,5',6,6'-terachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), selectively enters the mitochondria. In healthy cells, with high mitochondrial transmembrane potential ($\Delta\Psi m$), JC-1 forms J-aggregates with an intense red fluorescence at excitation/emission wavelength of 540/570 nm, respectively. In apoptotic or unhealthy cells, with low $\Delta\Psi m$, JC-1 remains in the monomeric form which fluoresces green at excitation/emission wavelength of 485/535 nm, respectively [Cheng *et al*., 2010]. Mitochondrial membrane potential was presented as the ratio of the J-aggregates (healthy cells) and monomeric forms (apoptotic cells). The experiment was performed in triplicate.

3.3.5.2. Phosphatidylserine externalization (Annexin V-FITC)

Mature biofilms were formed in Petri dishes and supplemented with PUFAs as described above. Biofilms were scraped off and washed with sterile PBS and the wet weight determined. Cells were pre-treated by resuspending the cells (4 mL/g wet

weight) in buffer (50 mM K_2HPO_4, 5 mM EDTA, 50 mM DTT, pH 7.2) and incubating at 30 ℃ for 30 minutes. Protoplast were formed by res uspending the cells (4 mL/g wet weight) in modified lysis buffer (50 mM K_2HPO_4, 40 mM ß-mercaptoethanol, 1.2 M sorbitol, 3 µg/mL chitinase, 100U/mL lyticase, 10000 U/mL ß-glucuronidase, 1000 U/mL zymolase 20T, pH 7.2) and incubating at 30 ℃ for 4 5 minutes. Formation of protoplasts was evaluated microscopically by SDS cell lysis. Protoplast were washed and resuspended in modified binding buffer (100 mM Hepes/NaOH, 1.2 M sorbitol, pH 7.5 containing 1.4 M Nacl, 25 mM $CaCl_2$) [Madeo *et al*., 1997; Phillips *et al*., 2003]. The Annexin-V binding assay for the detection of externalized phosphatidylserine [Annexin V-FITC Apoptosis Detection Kit (Sigma Aldrich, USA)], was performed by adding 5 µL Annexin V-FITC conjugate and 10 µL propidium iodide solutions to 500 µL of the cell suspensions and incubating for 10 minutes at room temperature. Cells were analyzed on the FACSCalibur Flowcytometer using CellQuest Pro software. Instrument-settings for standard human platelets were used to ensure the correct size particles were analyzed, as the yeast cells have approximately the same size as human platelets (2-3 µm in diameter).

3.3.5.3. DNA fragmentation (TUNEL) assay

For the analyses of DNA strand breaks, biofilms were prepared in Petri dishes and supplemented with PUFAs as described above. Cells were washed twice with PBS and fixed in 3.6 % formaldehyde. Fixed cells were washed twice with PBS and protoplasted as described and permeabilized in permeabilization solution (0.1 % Triton X-100 and 0.1% sodium citrate) for 2 minutes on ice. DNA strands were labeled with an *In Situ* Cell

Death Detection Fluorescein Kit (Roche Applied Science, Germany) at 37 ℃ for 1 hour [Philips *et al*., 2003]. Cells were analyzed on the FACSCalibur Flowcytometer using CellQuest Pro software. Instrument-settings for standard human platelets were used.

3.3.5.4. Nuclear condensation and fragmentation (DAPI staining)

Biofilms were prepared in chamber slides (Lab-Tek® Chamber Slide™ System, USA) and supplemented with PUFAs as described above. Nuclear condensation and fragmentation were analyzed by DAPI staining [Madeo *et al*., 1997]. Biofilms were washed twice with PBS, permeabilized in permeabilization solution (0.1 % Triton X-100 and 0.1% sodium citrate) for 2 minutes on ice, and incubated with 1 µg/mL of 4', 6-diamidino-2-phenylindole (DAPI) in the dark at 37 °C for 20 minutes [Hwang *et al*., 2011b]. Cells were examined by confocal laser microscopy.

3.3.6 Statistical analyses

Unless stated otherwise all experiments were conducted in triplicate on separate occasions. Averages and standard deviations were calculated and the student's *t*-test performed to determine the significance of the data sets. A *P* value of ≤ 0.01 was considered significant.

3.4. Results and Discussion

3.4.1. Phospholipid fatty acid analyses

The effect of supplementation of biofilms of *C. albicans* and *C. dubliniensis* grown in the absence and presence of PUFAs, on membrane phospholipid fatty acid composition is shown in Table 1 and Table 2, respectively. Figure 1 and Figure 2 indicate the unsaturation index of the phospholipids of both species, calculated for the different PUFAs. In this study, supplementation with C18:2 n-6 did not increase the unsaturation index for either species. This can be explained by the fact that C18:2 n-6 is part of the phospholipid fatty acid repertoire of these yeasts and is recognized and readily metabolized through β-oxidation. Interestingly, supplementation with C18:4 n-3 and C20:5 n-3, which were proposed to be inhibitory (Chapter 2), lead to the incorporation of these fatty acids into membrane phospholipids of both species, as highlighted in Table 1 and Table 2, and a resultant increase in unsaturation index (Fig. 1). However, C22:5 n-3 was poorly incorporated into the membrane phospholipids. This may be explained by the increased hydrophobicity of longer chain fatty acids.

Table 1: Fatty acid profiles of control biofilms of *C. albicans control biofilms* (A) and profiles following supplementation with C18:2 n-6 (B), C18:4 n-3 (C), C20:5 n-3 (D) and C22:5 n-3 (E). Values represent average of duplicate experiments.

(A) **Control**

Fatty Acid	Relative %
C16:0	26.4
C16:1 n-9	10.6
C18:0	13.4
C18:1 n-9	35.3
C18:2 n-6	13.1
C18:3 n-3	1.2
C20:0	0.1

(B) **C18:2 n-6**

Fatty Acid	Relative %
C16:0	30.9
C16:1 n-9	3.4
C18:0	15.7
C18:1 n-9	24.4
C18:2 n-6	22.9
C18:3 n-3	1.1
C20:0	0.3
C20:2 n-6	3.3
C20:5 n-3	0.3

(C) **C18:4 n-3**

Fatty Acid	Relative %
C16:0	24.9
C16:1 n-9	2.4
C18:0	19.2
C18:1 n-9	14.3
C18:2 n-6	6.2
C18:3 n-3	0.7
C18:4 n-3	33.6
C20:0	0.3
C20:5 n-3	0.4

(D) **C20:5 n-3**

Fatty Acid	Relative %
C16:0	14.4
C16:1 n-9	0.9
C18:0	8.6
C18:1 n-9	17.6
C18:2 n-6	6.0
C18:3 n-3	0.6
C20:0	0.1
C20:1 n-9	0.3
C20:2 n-6	0.1
C20:3 n-3	0.3
C20:4 n-6	0.2
C20:5 n-3	51.5

(E) **C22:5 n-3**

Fatty Acid	Relative %
C16:0	29.4
C16:1 n-9	5.8
C18:0	14.8
C18:1 n-9	32.5
C18:2 n-6	10.0
C18:3 n-3	1.5
C20:0	0.3
C20:3 n-3	0.1
C20:5 n-3	3.0
C22:5 n-3	5.0

Table 2: Fatty acid profiles of control biofilms of *C. dubliniensis* control biofilms (A) and profiles following supplementation with C18:2 n-6 (B), C18:4 n-3 (C), C20:5 n-3 (D) and C22:5 n-3 (E). Values represent average of duplicate experiments.

(A) **Control**

Fatty Acid	Relative %
C16:0	34.8
C16:1 n-9	5.3
C18:0	23.5
C18:1 n-9	27.0
C18:2 n-6	8.6
C18:3 n-3	0.9
C20:0	0.6
C20:5 n-3	0.3

(B) **C18:2 n-6**

Fatty Acid	Relative %
C16:0	38.8
C16:1 n-9	4.2
C18:0	18.1
C18:1 n-9	17.5
C18:2 n-6	22.6
C18:3 n-3	1.1
C20:0	0.4
C20:2 n-6	0.1
C20:5 n-3	0.3

(C) **C18:4 n-3**

Fatty Acid	Relative %
C16:0	28.9
C16:1 n-9	1.7
C18:0	20.4
C18:1 n-9	17.5
C18:2 n-6	8.5
C18:3 n-3	0.7
18:4 n-3	21.9
C20:0	0.6
C20:5 n-3	0.5

(D) **C20:5 n-3**

Fatty Acid	Relative %
C16:0	22.8
C16:1 n-9	1.3
C18:0	13.6
C18:1 n-9	14.6
C18:2 n-6	7.6
C18:3 n-3	0.6
C20:0	0.4
C20:5 n-3	40.2

(E) **C22:5 n-3**

Fatty Acid	Relative %
C16:0	29.2
C16:1 n-9	3.0
C18:0	21.1
C18:1 n-9	31.4
C18:2 n-6	9.7
C18:3 n-3	1.1
C20:0	0.3
C20:1 n-9	0.1
C20:2 n-6	0.1
C20:4 n-6	0.2
C20:5 n-3	2.6
C22:5 n-3	3.0

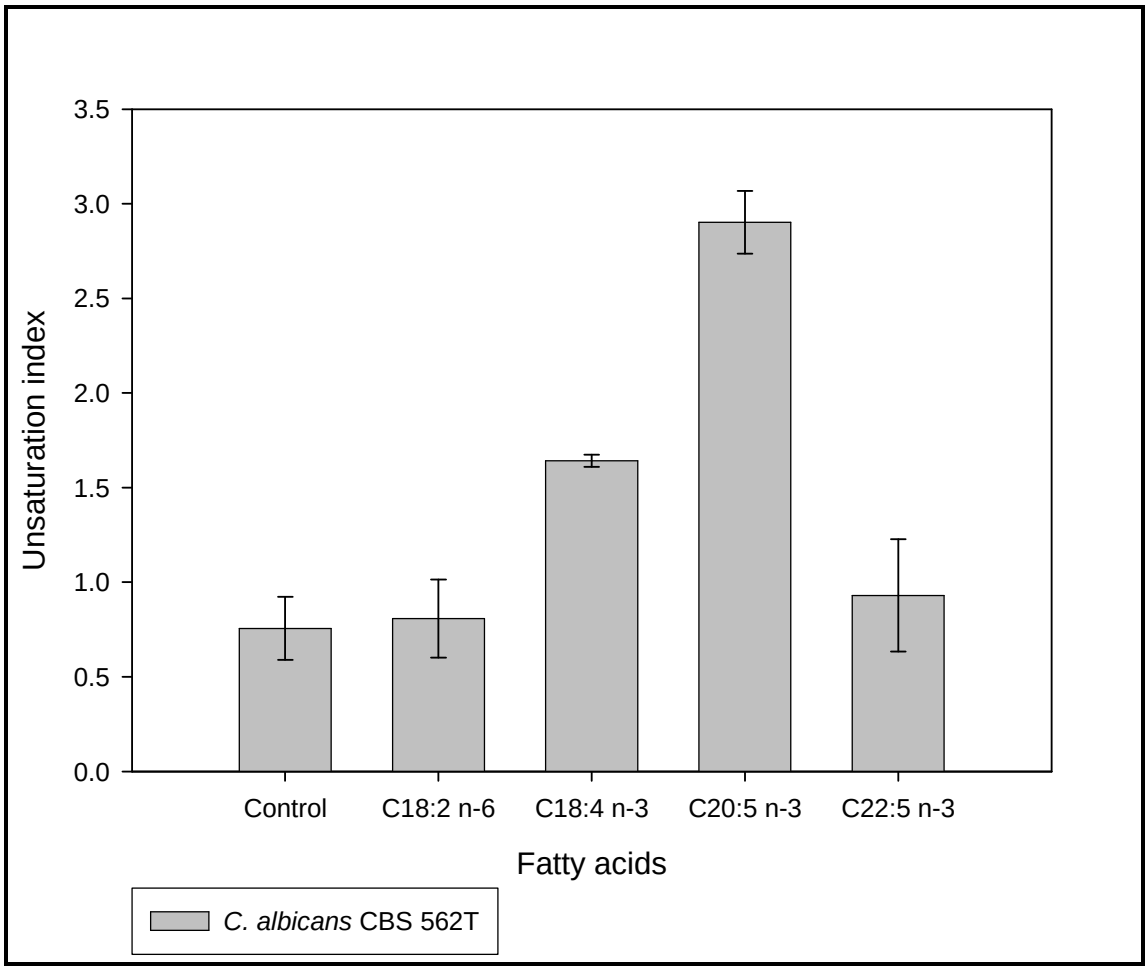

Fig. 1: Unsaturation index of biofilms of *C. albicans* following supplementation by C18:2 n-6, C18:4 n-3, C20:5 n-3 and C22:5 n-3 compared to unsupplemented biofilm. Values are the averages of duplicate experiments and error bars indicate the range.

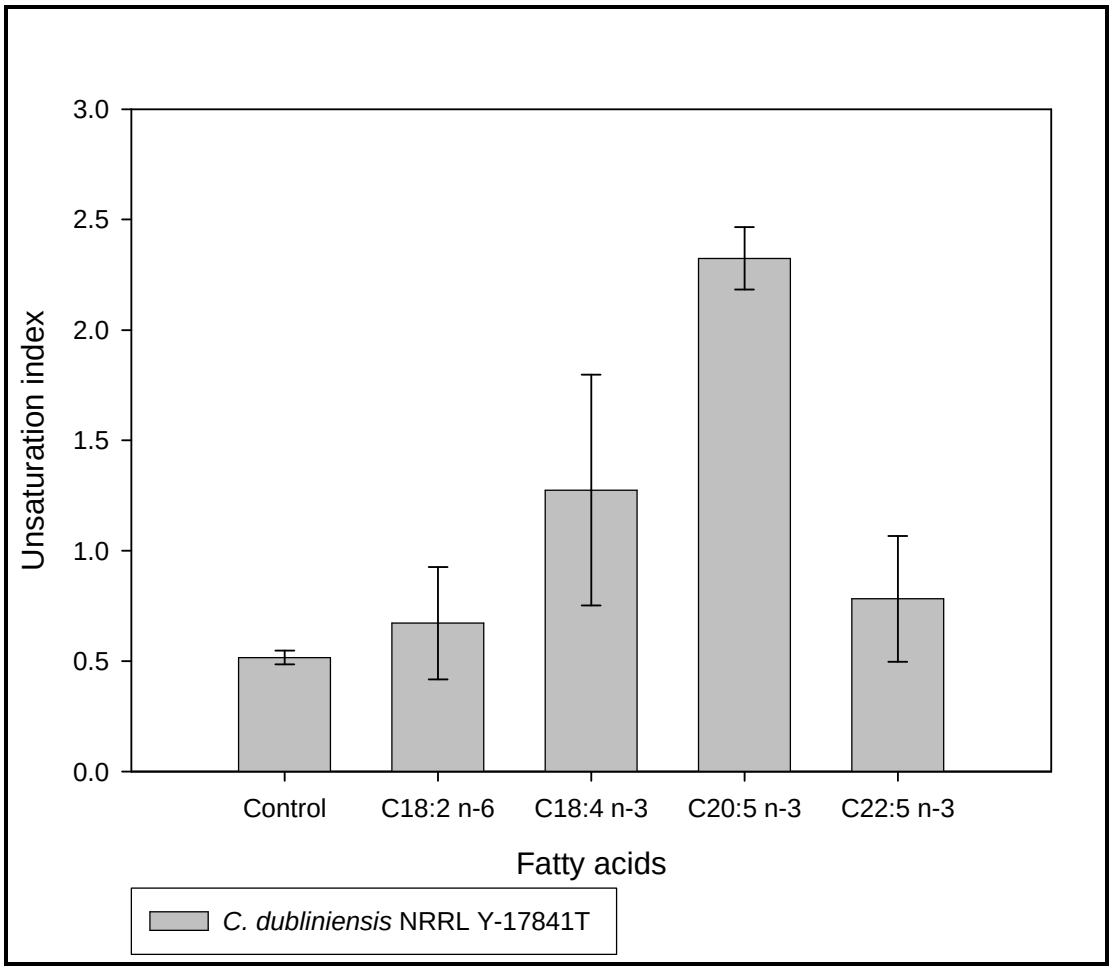

Fig. 2: Unsaturation index of biofilms of *C. dubliniensis* following supplementation by C18:2 n-6, C18:4 n-3, C20:5 n-3 and C22:5 n-3 compared to unsupplemented biofilm. Values are the averages of duplicate experiments and error bars indicate the range.

3.4.2. Oxidative stress

Figure 3 depicts the response of *C. albicans* and *C. dubliniensis* biofilms to oxidative stress after supplementation with PUFAs. There was a significant increase ($P \leq 0.01$) in ROS production when biofilms by *C. albicans* (Fig. 3A) were supplemented with C18:4 n-3, an indication of oxidative stress. Furthermore, there was no significant difference when biofilms by *C. albicans* were with C18:2 n-6, C20:5 n-3 and C22:5 n-3. When *C.*

dubliniensis biofilms (Fig. 3B) were supplemented with C18:4 n-3 and C20:5 n-3, there was a significant increase ($P \leq 0.01$) in ROS production. However, when *C. dubliniensis* was supplemented with C18:2 n-6 and C22:5 n-3 there was no significant increase in ROS production. The results indicated that supplementation with both C18:4 n-3 and C20:5 n-3 could potentially increase oxidative stress in biofilms by *C. albicans* and *C. dubliniensis*. Increased production of ROS leads to an increase in radical by-products that could attack membrane phospholipid and induce lipid peroxidation. Figure 3C and 3D show increased concentrations of malondialdehyde, a biomarker of lipid peroxidation, in biofilms by *C. albicans* and *C. dubliniensis* following fatty acid supplementation, respectively. Interestingly, although there was a significant increase in ROS production with only some fatty acids, there was an increase in lipid peroxidation following supplementation with all selected fatty acids in both *C. albicans* and *C. dubliniensis*. This might suggest that other pathways other than oxidative stress via ROS production may be involved in the induction of lipid peroxidation.

The observed increased concentration of malondialdehyde with all the selected fatty acids might occur as a result of increased levels of lipid peroxides, following abstraction of hydrogen atoms from membrane phospholipids, lipid peroxidation, by radical species. The formed lipid peroxides can further abstract hydrogen atoms from membrane phospholipids resulting in increased concentration of malondialdehyde.

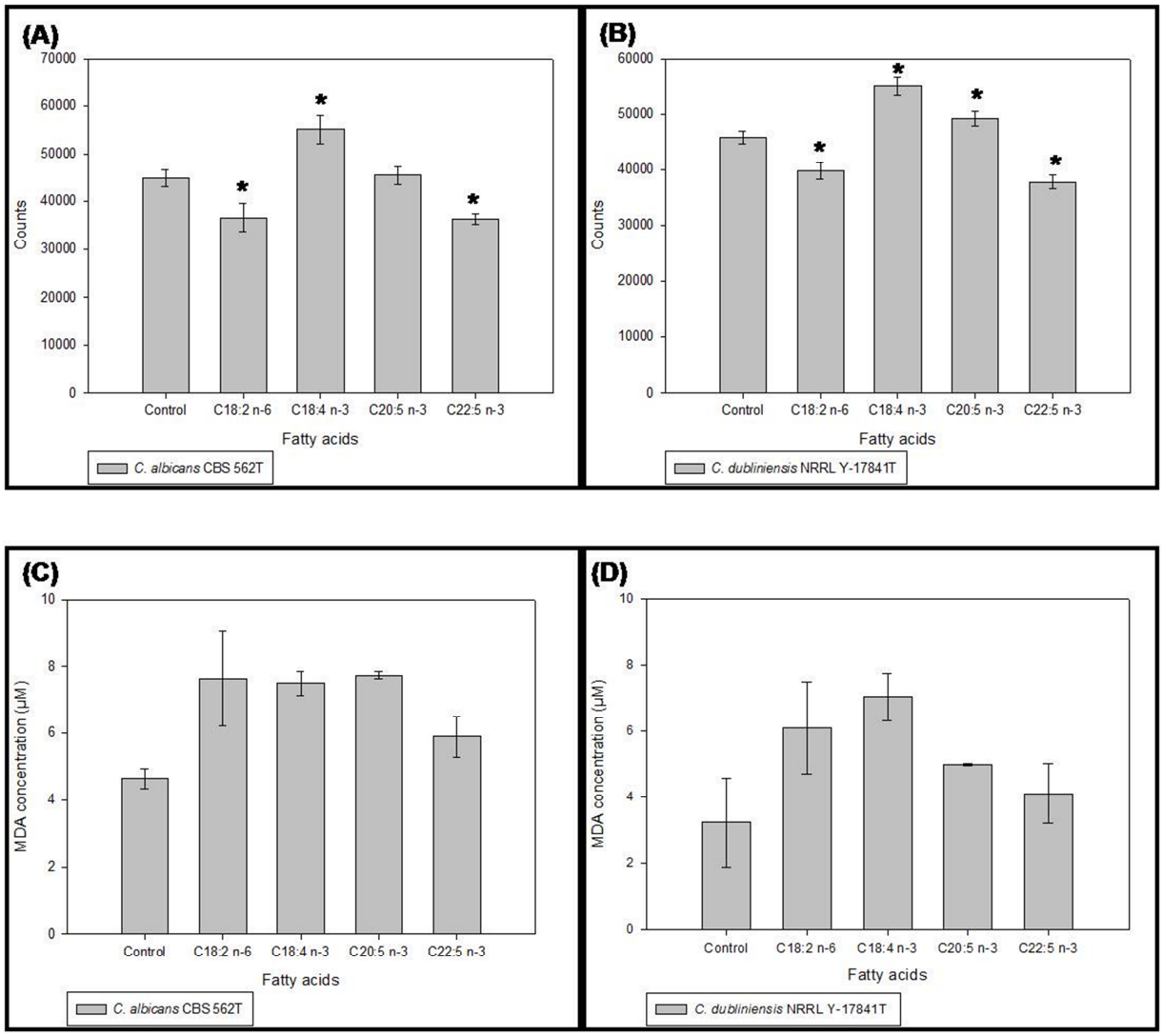

Fig. 3: Oxidative stress in biofilms of *C. albicans* and *C. dubliniensis*. Accumulation of ROS following PUFA supplementation of *C. albicans* (A) and *C. dubliniensis* (B). Values are averages of triplicate experiments and error bars indicate the standard deviation. * = significantly different from control ($P \leq 0.01$). Malondialdehyde (MDA), concentration of *C. albicans* (C) and *C. dubliniensis* (D) biofilms. Values are averages of duplicate experiments and error bars indicate the range.

3.4.3. Apoptotic markers

3.4.3.1. Mitochondrial transmembrane potential

The loss of mitochondrial transmembrane potential (ΔΨm) is one of several key events that occur in the mitochondria during early stages of apoptosis [Green & Reed, 1998]. There was a significant loss ($P \leq 0.01$) in ΔΨm when biofilms of *C. albicans* were supplemented with C18:4 n-3, C20:5 n-3 and C22:5 n-3 (Fig. 4). However, supplementation of the biofilm with C18:2 n-6 did not have a significant effect compared to the control, the ΔΨm remain intact. This correlates with the non-inhibitory nature of C18:2 n-6 on biofilms of *C. albicans*. The accumulation of ROS and other reactive metabolites are known to induce rapid loss of ΔΨm [Fleury *et al*, 2002].

Our previous results (Chapter 2) have shown the *C. dubliniensis* strain to be more resistant to PUFAs supplementation and was also more resistant to loss of ΔΨm compared to *C. albicans*. Although supplementation of *C. dubliniensis* biofilms with C18:4 n-3 and C20:5 n-3 resulted in a significant increase in ROS production (Fig. 3B) and supplementation with C18:4 n-3 resulted in a significant loss in ΔΨm (Fig. 5), there were no significant differences to the control when C18:2 n-6, C20:5 n-3 and C22:5 n-3 were used in supplementation. The results indicate that supplementation with certain PUFAs induces early apoptosis in biofilms of both *C. albicans* and *C. dubliniensis*.

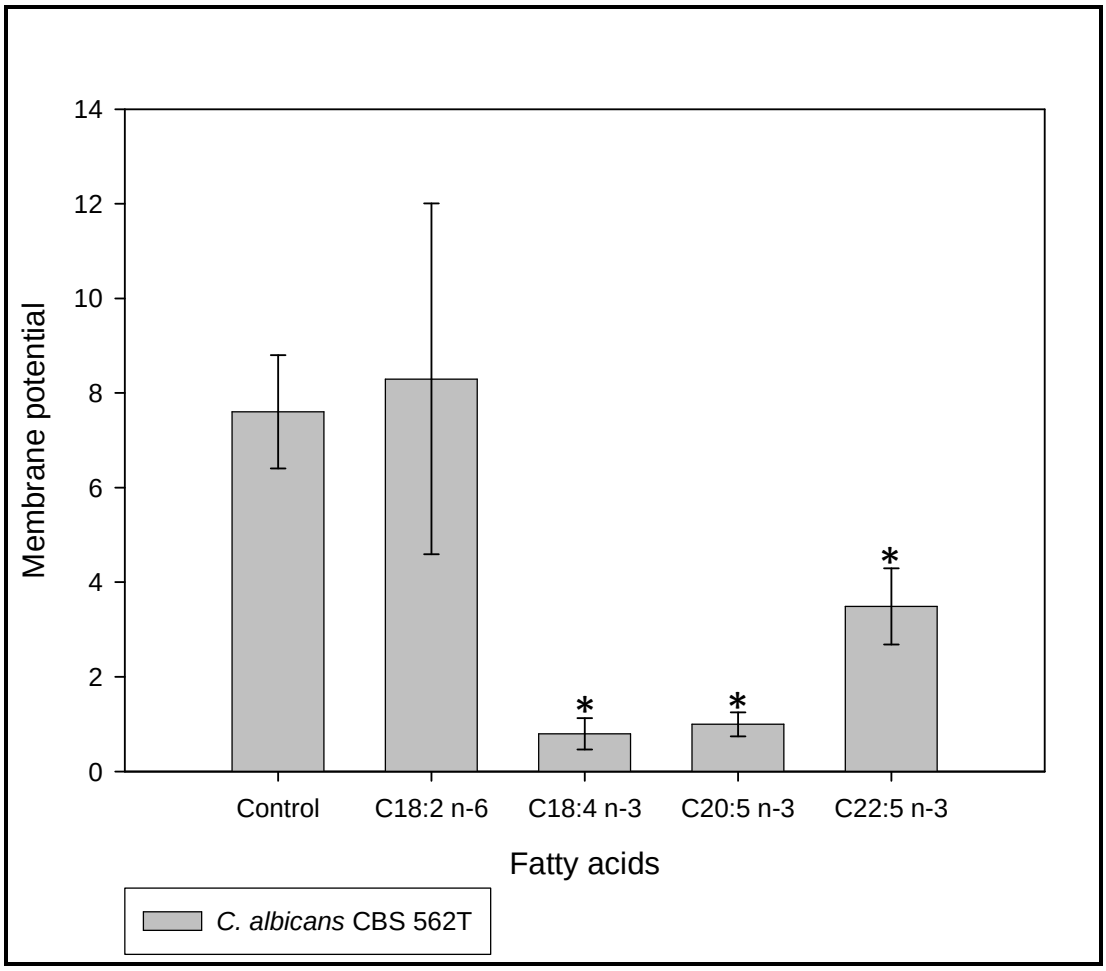

Fig. 4: Mitochondrial transmembrane potential (ΔΨm) of biofilms of *C. albicans,* presented as the ratio of the J-aggregates (healthy cells) and monomeric forms (apoptotic cells). A decrease in the ratio serves as an indication of loss of mitochondrial membrane potential. Values are averages of triplicate experiments and error bars indicate the standard deviation. * = significantly different from control ($P \leq 0.01$).

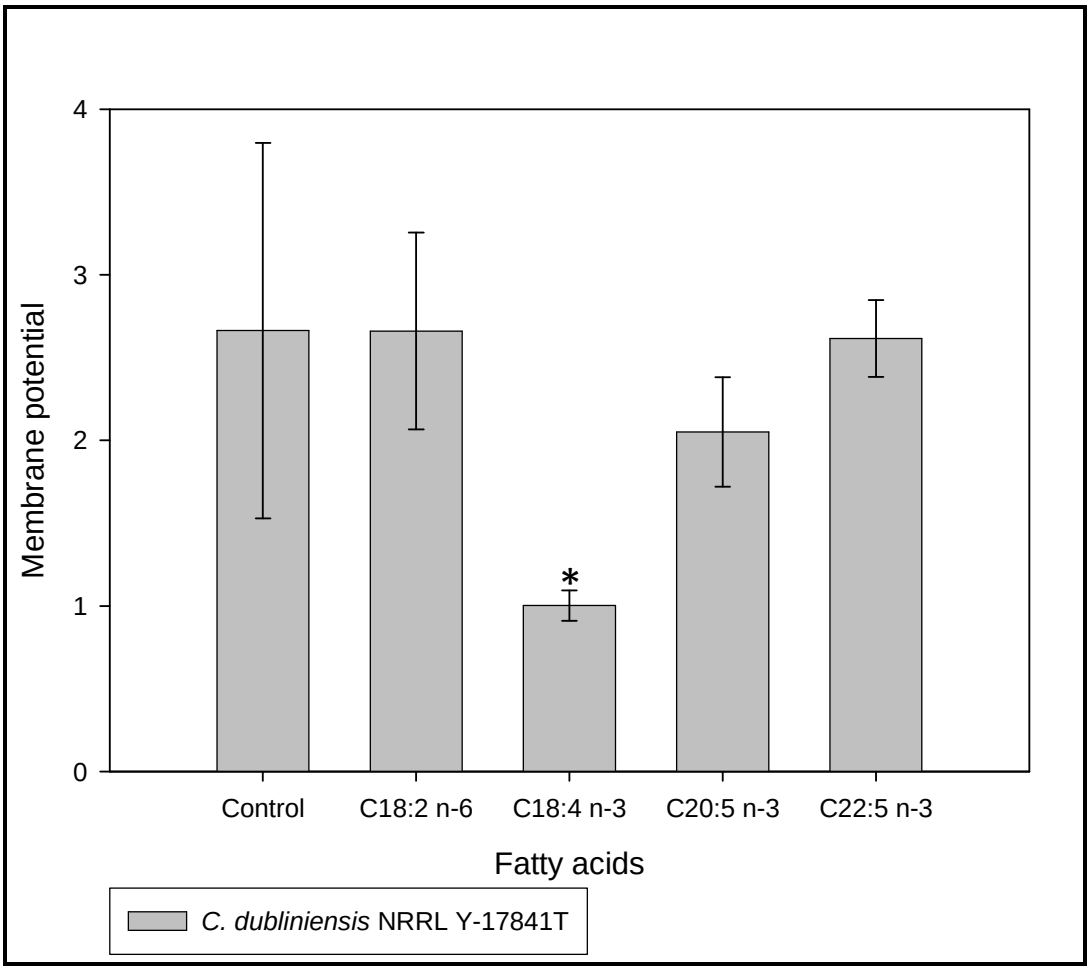

Fig. 5: Mitochondrial membrane potential (ΔΨm) of biofilms of *C. dubliniensis,* presented as the ratio of the J-aggregates (healthy cells) and monomeric forms (apoptotic cells). A decrease in the ratio serves as an indication of loss of mitochondrial membrane potential. Values are averages of triplicate experiments and error bars indicate the standard deviation. * = significantly different from control ($P \leq 0.01$).

3.4.3.2. Phosphatidylserine externalization (Annexin V-FITC)

To differentiate between apoptosis and necrosis, the Annexin V-FITC and propidium iodide (PI) double staining method was used in this study. Annexin V has a high affinity for PS in the presence of calcium ions (Ca^{2+}) and it can be used to detect PS externalization, an indication of early stages of apoptosis. Propidium iodide will be taken through the permeabilized plasma membrane and will accumulate in the nucleus of necrotic cells [Madeo *et al*, 1997]. The results for *C. albicans* (Fig. 6) showed that supplementation with C20:5 n-3 resulted in the highest percentage of cells in early stages of apoptosis while supplementation with C22:5 n-3 showed the lowest percentage of cells in early apoptosis. Interestingly, even though C18:2 n-6 was proposed to be non-inhibitory, supplementation with the fatty acid resulted in a significant percentage of cells in early stages of apoptosis. There was not a observed difference in the percentage cells of *C. dubliniensis* (Fig. 6) going into early apoptosis between all PUFAs used. The results from this study showed that supplementation with PUFAs induces early stages of apoptosis in biofilms of both *C. albicans* and *C. dubliniensis*.

3.4.3.3. DNA fragmentation (TUNEL) assay

The DNA strand break into fragmentation is an indication of late stages of apoptosis. The TUNEL assay is a common method for detecting the cleavage of double stranded apoptotic DNA in individual nuclei by labeling fluorescent dUTP at 3'-OH end of DNA [Madeo *et al*., 1997]. The study evaluated if biofilms of *C. albicans* and *C. dubliniensis*

supplemented with PUFAs display features of late apoptosis. The results indicated a significant increase ($P \leq 0.01$) in percentage of cells going into late stages of apoptosis during supplementation with all PUFAs in both *C. albicans* and *C. dubliniensis* (Fig. 6). The highest percentage of cells in late apoptosis was obtained after supplementation with C18:4 n-3 and C20:5 n-3. For both species, the lowest percentage cells in late apoptosis was obtained after supplementation with C18:2 n-6.

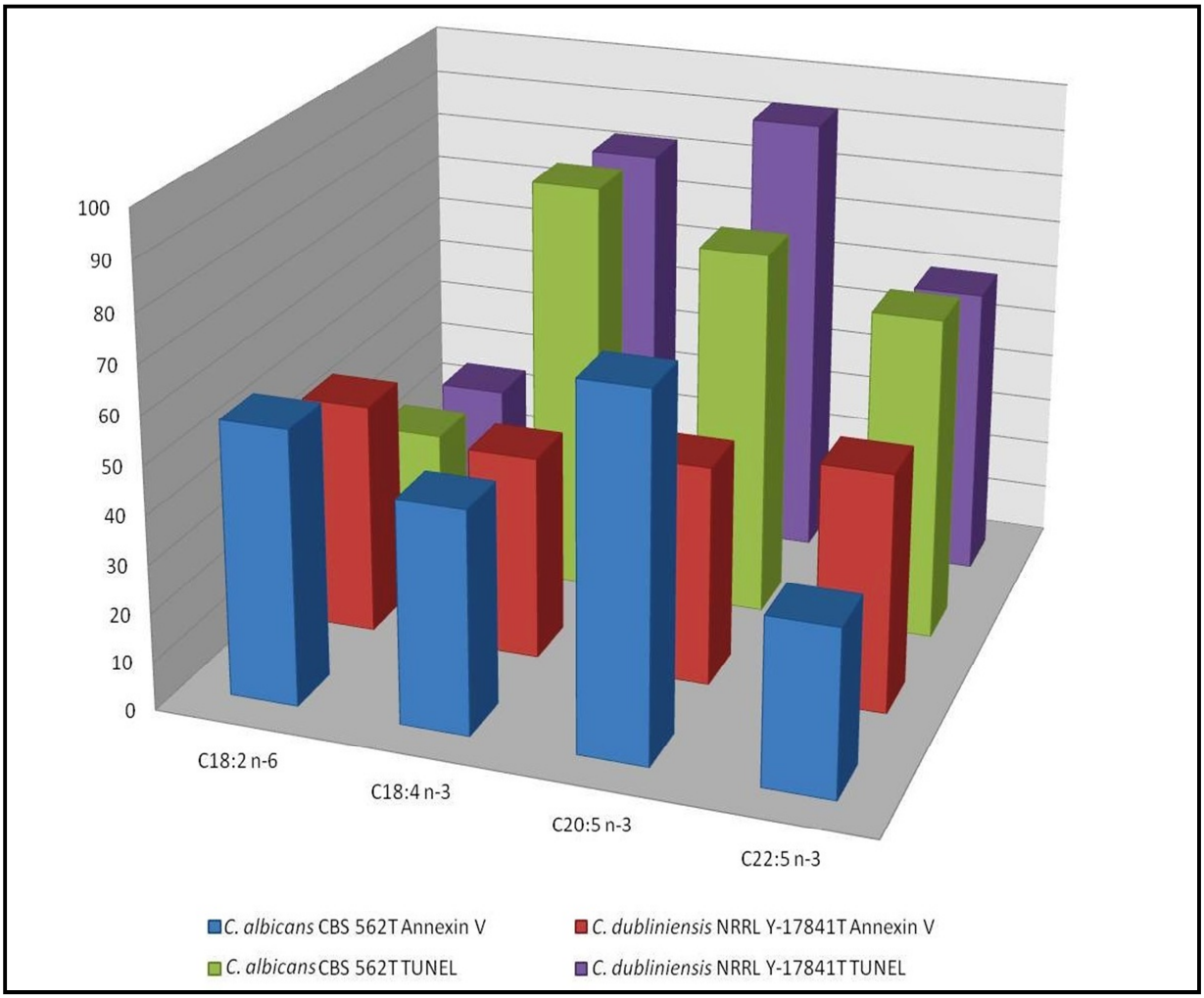

Fig. 6: Percentage of cells displaying various apoptotic markers. The graph depicts the externalization of phosphatidylserine (PS), an indication of early stages of apoptosis, through Annexin V-FITC analyses of biofilms of *C. albicans* and *C. dubliniensis*. The graph also depicts DNA fragmentation, an indication of late stages of apoptosis, observed using the TUNEL assay method.

3.4.3.4. Nuclear condensation and fragmentation (DAPI staining)

The DAPI stain, a cell permeable fluorescent dye, is commonly used to detect late stages of apoptosis by showing nuclear condensation and fragmentation [Madeo *et al*., 1997]. The stain binds strongly and selectively to the minor grooves of adenine and thymine-rich sequences of DNA and show fold increase in fluorescence intensity compared to unbound DAPI [Kapuscinski, 1995]. During this study, DAPI staining was used in combination with the TUNEL assay to emphasize the effect PUFAs have in inducing late apoptosis in biofilms of *C. albicans* and *C. dubliniensis*. Figure 7 shows an increase in fluorescence intensity of biofilms of *C. albicans*, showing nuclear condensation and fragmentation, following supplementation with C18:4 n-4, C20:5 n-3 and C22:5 n-3. However, there was no observed increase in fluorescence intensity with control biofilms and biofilms supplemented with C18:2 n-6 an indication of absence of late apoptosis.

Furthermore, similar results were obtained against *C. dubliniensis* (Fig. 8) with control biofilms and biofilms supplemented with C18:2 n-6 showing lower fluorescence intensity, indicative of absence of late apoptosis. However, there was an observed increase in fluorescence intensity in biofilms supplemented with C18:4 n-3, C20:5 n-3 and C22:5 n-3, an indication of late apoptosis. The results from this study showed C18:4 n-3, C20:5 n-3 and C22:5 n-3 to inhibit growth of biofilms of both *C. albicans* and *C. dubliniensis*, inducing late apoptosis.

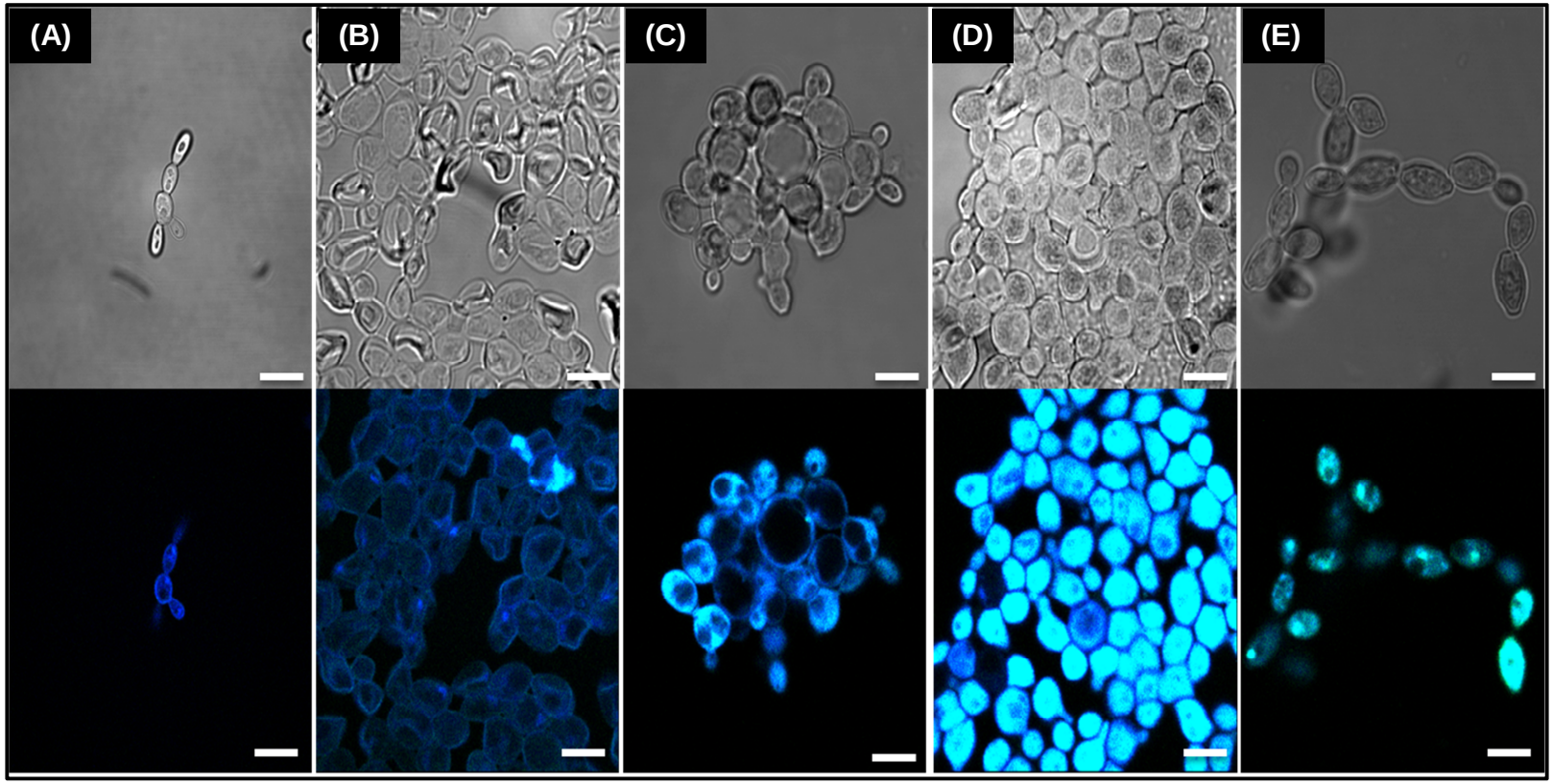

Fig. 7: Nuclear condensation and fragmentation of *C. albicans* biofilms visualized by fluorescence microscopy using DAPI staining. Control biofilms (A) and biofilms supplemented with C18:2 n-6 (B) show less intense fluorescence compared to biofilms supplemented with C18:4 n-3 (C), C20:5 n-3 (D) and C22:5 n-3 (E). Scale bar, 5 µm.

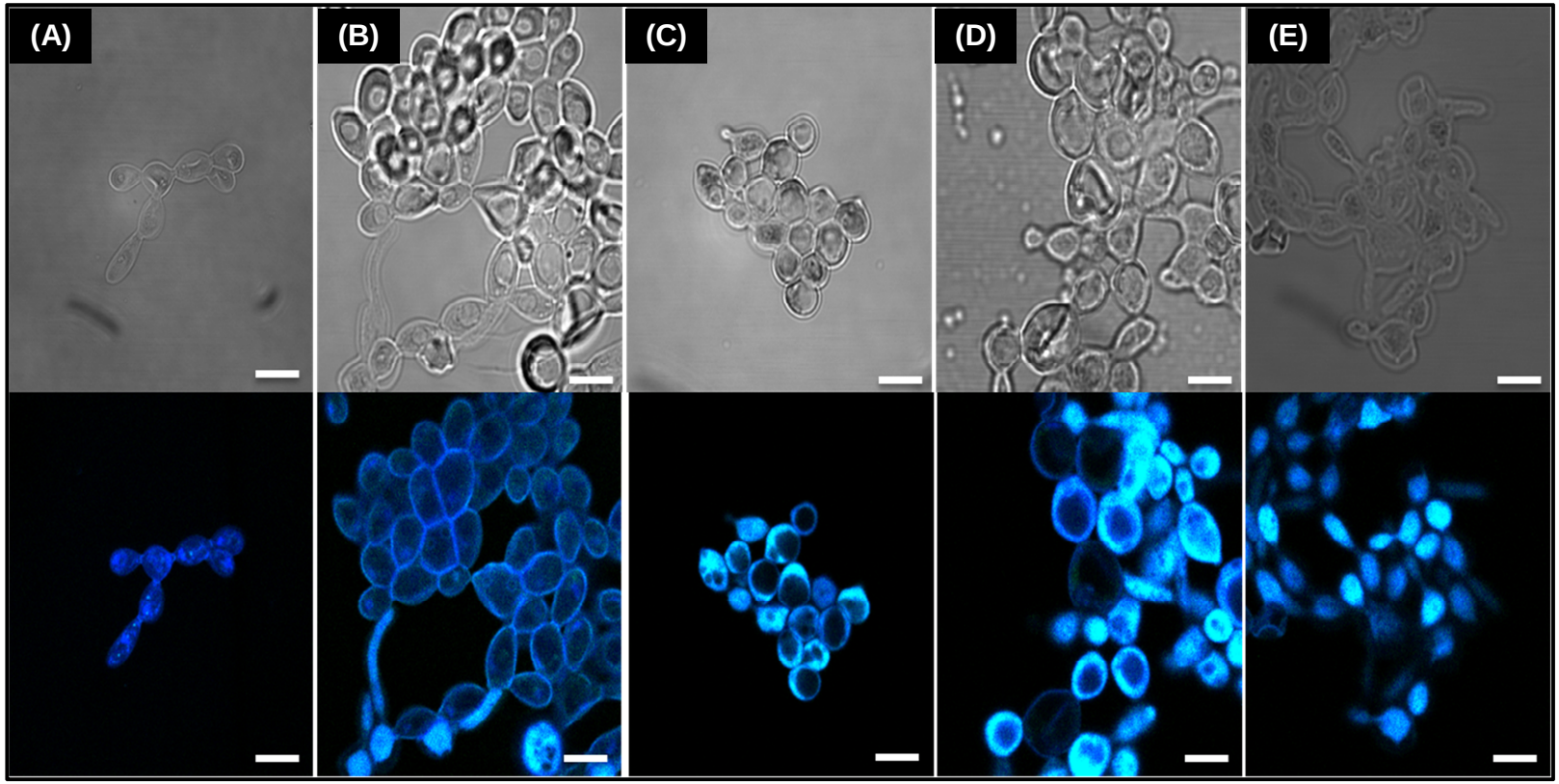

Fig. 8: Nuclear condensation and fragmentation of *C. dubliniensis* biofilms visualized by fluorescence microscopy using DAPI staining. Control biofilms (A) and biofilms supplemented with C18:2 n-6 (B) show less intense fluorescence compared to biofilms supplemented with C18:4 n-3 (C), C20:5 n-3 (D) and C22:5 n-3 (E). Scale bar, 5 µm.

3.5. Conclusions

Saturated, monounsaturated and polyunsaturated fatty acids are readily taken up and incorporated into the membrane phospholipids. But the degrees to which these fatty acids interact with the membrane differ because of variations in length and saturation [Liu *et al*., 2008]. Supplementation of *C. albicans* and *C. dubliniensis* biofilms with C18:4 n-3 and C20:5 n-3 increased the unsaturation index of cell membranes. We found that, especially C18:4 n-3, C20:5 n-3 and C22:5 n-3 could cause apoptosis as determined with the TUNEL assay and DAPI staining, probably by causing an increase in ROS production and loss of ΔΨm. The most effective PUFA in this regard against both species was C18:4 n-3.

The development of resistance towards antifungal agents already in use makes it necessary to search for novel antifungals, including fatty acids. Although fatty acids may not be as effective as chemical antifungals, they exhibit a high degree of specificity and pathogenic fungi are less likely to become resistant to antifungal fatty acids [Pohl *et al.*, 2011]. Although most of the research regarding the health benefits of C18:4 n-3 has focused on dietary supplementation, another antifungal fatty acid, undecylenic acid, has successfully been applied in treatment as a topical preparation [Li *et al.*, 2008], paving the way for the use of other antifungal fatty acids in the prevention and possible treatment of fungal biofilm related infections. Interestingly, this fatty acid was also found to be the antifungal agent in denture liners used to treat *C. albicans* associated denture somatitis [McLain *et al.,* 2000]. It may therefore be of interest to study weather incorporation of C18:4 n-3 into devices such as denture liners or catheters may prevent biofilm formation by these pathogenic yeasts. In addition, previous studies [Ells *et al.*,

2009] have indicated that the PUFA, arachidonic acid, may increase the susceptibility of *C. albicans* and *C. dubliniensis* biofilms to traditional antifungals, so lowering the dose of these antifungals needed. The effect of C18:4 n-3 in this regard will be studied in the next chapter.

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Chapter 4

Interaction of amphotericin B and stearidonic acid in inhibition of biofilm formation by *C. albicans* and *C. dubliniensis*

4.1. Summary

The increasing incidences of drug-resistant pathogens and toxicity of existing antifungal compounds has drawn attention to the antimicrobial activity of natural products. New antifungal agents are urgently required to combat life threatening infections caused by fungal pathogens like *Candida* species. Combination therapy is an important strategy, as synergistic interactions can potentially increase antifungal efficiency compared to monotherapy. Therefore, the aim of the current study was to determine potential synergistic interaction that exists between amphotericin B (AmB) and stearidonic acid (C18:4 n-3), an antifungal polyunsaturated fatty acid, on biofilm formation by *C. albicans* and *C. dubliniensis*.

The fractional inhibitory concentration index (FICI) analyses were used to determine the antifungal interaction (which can either be synergistic, additive/indifferent or antagonistic) of AmB and C18:4 n-3 when used in combination. The interaction was determined to be synergistic against both *C. albicans* and *C. dubliniensis* with an FICI value of 0.28 and 0.38, which correlated to inhibition of 99 and 96% respectively. The results are an indication that lowered dosages of antifungal compounds can be used in synergism with antifungal polyunsaturated fatty acids to combat fungal infections.

4.2. Introduction

The development of resistance of fungi towards antifungal compounds remains high, despite significant progress made in the combat of infectious diseases caused by fungal pathogens [Groll & Walsh, 2002]. As an example, *Candida* biofilms are known to be resistant to a range of antifungal agents in current clinical use [Al-Dhaheri & Douglas, 2010]. Furthermore, antifungal compounds that are toxic to fungi also have undesirable side effects on mammalian hosts and their toxicity delays antifungal drug discovery [Okoli *et al*., 2009]. As a result, attention has been drawn towards finding alternative therapeutic methods [Phillips *et al*., 2003; Yang *et al*., 2010]. Combination therapy is an important method in this regard, as synergistic and additive interactions can potentially increase antifungal efficiency by providing a broader-spectrum of antifungal activity, preventing the emergence of resistance and reducing toxicity, compared to monotherapy [Marr *et al*., 2004].

Amphotericin B (AmB) is an important antifungal compound widely used to treat systemic mycoses. It is generally accepted that AmB binds to the ergosterol of fungal cell membranes, increasing membrane permeability and eventually killing the cell [Brajtburg, *et al*., 1990]. In addition, several studies have demonstrated the involvement of oxidative damage induced by reactive oxygen species (ROS) in AmB antifungal activity against *Candida albicans* [Sokol-Anderson *et al*., 1986; Okamoto *et al*., 2004]. However, owing to poor permeability of AmB across membranes, an increased dosage must be administered, which often leads to severe side effects [Fanos & Cataldi, 2000]. Therefore, an investigation in reducing the AmB dosage by combining it with other compounds is necessary and the antimicrobial activity of natural products, such as fatty

acids, may be the answer. Polyunsaturated fatty acids are known to have antifungal properties [Liu *et al*., 2008] and in our recent work, we showed that C18:4 n-3 has antifungal properties, inhibiting biofilm formation and inducing apoptosis in *C. albicans* and *C. dubliniensis* (Chapter 2 and Chapter 3). Therefore, the aim of this study was to examine the potential interaction that exists between AmB and C18:4 n-3 on biofilm formation by *C. albicans* and *C. dubliniensis*.

4.3. Materials and methods

4.3.1. Strains and antifungals used

The antifungal activity of AmB, dissolved in dimethyl sulfoxide (DMSO), and C18:4 n-3, dissolved in ethanol, were tested against *Candida albicans* CBS 562T and *Candida dubliniensis* NRRL Y-17841T. Both strains were maintained on yeast malt extract (YM) agar plates (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. The strains were also stored on agar slants at 4 ℃.

4.3.2. Biofilm formation

Cells of *C. albicans* and *C. dubliniensis* were grown separately on YM agar plates and incubated at 30 ℃ for 24 hours. After incubation, a loop-full of the cells was inoculated into 20 mL of yeast nitrogen base (YNB) glucose medium (10 g/L glucose, 6.7 g/L YNB) and incubated at 30 ℃ for 48 hours. Cells were washed twice with sterile phosphate buffered saline (PBS) and diluted in filter sterilized RPMI-1640 medium (Sigma-Aldrich, UK) to a standardized cell concentration of 1×10^6 cells/mL. A volume of 100 µL of the

standardized cell suspension was dispensed into a 96-well microtiter plate (Corning Incorporated, Costar®, U.S.) and further incubated at 37 ℃ for 1 hour to allow adherence of cells to the surface [Samaranayake *et al*., 1995]. Wells were washed twice with PBS to remove non-adherent cells.

4.3.3. Determination of minimum inhibitory concentration (MIC)

Serial dilutions of the antifungal compounds, AmB and C18:4 n-3 were prepared in filter sterilized RPMI-1640 medium. The concentrations of the AmB ranged from 100 mg/L to 0.01 mg/L (tenfold dilution) and those of C18:4 n-3 from 1 mM to 62.5 µM (twofold dilution), for the determination of their MIC values. Amphotericin B and C18:4 n-3 were added to the 1 hour old biofilm prepared as described above and mature biofilms were formed by incubating at 37 ℃ for an additional 47 hours. The reduction of (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (XTT) (Sigma Aldrich, UK) in the presence of metabolic activity was used to examine yeast viability. XTT is converted to formazan which is colored and easily measured in cellular supernatants in terms of optical density at a wavelength of 492 nm [Kuhn *et al*., 2003]. This experiment was done in triplicate.

4.3.4. Checkerboard assay

Serial twofold dilutions of C18:4 n-3 and AmB were prepared from at least double the determined MIC in RPMI-1640 medium. The concentrations of both the DMSO and ethanol never exceeded 2 %. Two 96-well plates were prepared. The first plate was

used to make twofold serial dilutions of C18:4 n-3 in a horizontal orientation. The second plate was used to make twofold serial dilutions of AmB in a vertical orientation. A volume of 50 µL of each of the two dilution series were combined and added to the 1 hour old biofilm prepared as described above and mature biofilm were then formed by incubating at 37 ℃ for an additional 47 hours and metabolic activity was measured using the XTT assay [Orhan *et al*., 2005]. This experiment was done in triplicate.

4.3.5. Fractional inhibitory concentration index (FICI) analyses

The results of the combined effects of AmB and C18:4 n-3 were calculated and expressed in terms of fractional inhibitory concentration index (FICI). The FICI was calculated in each well of the microtiter plate and the concentrations of AmB and C18:4 n-3 corresponding to the well were used in the calculations. The equation, $FICI = FIC_A + FIC_B = [(C_A/MIC_A) + (C_B/MIC_B)]$ where MIC_A and MIC_B are the MIC's of AmB and C18:4 n-3 alone respectively and C_A and C_B are the concentrations of AmB and C18:4 n-3 used in combination. The results were considered synergistic when FICI was ≤ 0.5, as additive/indifferent if FICI was between 0.5 and 4 or as antagonistic when FICI was >4 [Meletiadis *et al*., 2005].

4.4. Results and Discussion

4.4.1. Determination of minimum inhibitory concentration (MIC)

The percentage inhibition of metabolic activity by AmB on biofilm formation of *C. albicans* and *C. dubliniensis* is shown in Table 1. The MIC for *C. albicans* was chosen to

be 10 mg/L. Since complete inhibition was not observed for *C. dubliniensis*, the lowest concentration which resulted in at least 99% was selected (10 mg/L). Interestingly, even at concentration 100 folds lower (0.1 mg/L) than the MIC, significant inhibition was observed against *C. albicans*.

Table 2 shows percentage inhibition of metabolic activity by C18:4 n-3 on biofilm formation by *C. albicans* and *C. dubliniensis*. The MIC for both *C. albicans* and *C. dubliniensis* was selected to be 1 mM since it resulted in the highest % inhibition even though complete inhibition was not observed. The results showed that *C. dubliniensis* was more resistant to supplementation by C18:4 n-3. It is interesting to note that the lowest concentration used (62.5 µM) against *C. albicans* was able to inhibit biofilm formation by about 50 %. The resistance of the *C. dubliniensis* strain used in this study, towards lower doses of antifungal compounds might be explained by its ability to form dense network of hyphae, an ability that was absent in the more susceptible *C. albicans* strain used in the study.

Table 1: Percentage inhibition of metabolic activity by amphotericin B (AmB) supplementation of *C. albicans* and *C. dubliniensis*. Positive values indicate reduced reduction of the XTT salt and negative values indicates increased reduction of the XTT salt. Values are the averages of triplicate values with the standard deviation indicated in the brackets.

	Amphotericin B (AmB)			
	C. albicans CBS 562T		*C. dubliniensis* NRRL Y-17841T	
100mg/L	100	(1.07)	99.61	(0.88)
10mg/L	100	(0.67)	99.29	(0.45)
1mg/L	99.19	(0.4)	98.09	(0.63)
0.1mg/L	64.88	(2.44)	-7.63	(6.24)
0.01mg/L	-4.98	(2.57)	-17.02	(6.84)

Table 2: Percentage inhibition of metabolic activity by stearidonic acid (C18:4 n-3) supplementation on *C. albicans* and *C. dubliniensis*. Positive values indicate reduced reduction of the XTT salt and negative values indicates increased reduction of the XTT salt. Values are the averages of triplicate values with the standard deviation indicated in the brackets.

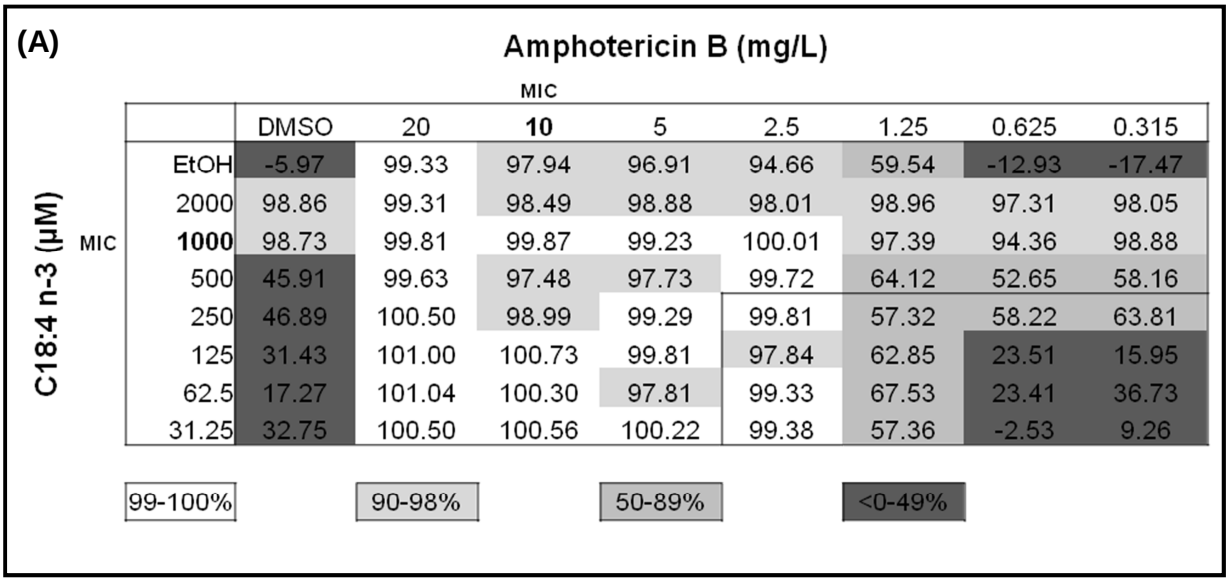
	Stearidonic acid (C18:4 n-3)			
	C. albicans CBS 562T		*C. dubliniensis* NRRL Y-17841T	
1mM	97.01	(1.19)	98.35	(1.09)
500µM	81.32	(4.11)	-4.59	(16.07)
250µM	62.19	(4.43)	-12.49	(1.54)
125µM	56.47	(16.06)	-19.89	(12.51)
62.5µM	49.58	(10.82)	-34.39	(12.47)

4.4.2. Checkerboard assay

The percentage inhibition of *C. albicans* (Fig. 1A) and *C. dubliniensis* (Fig. 2A) with different concentration combinations vary from partial to total inhibition. The minimum FICI found to be synergistic was 0.28 and 0.38 for *C. albicans* and *C. dubliniensis* (Fig. 1B, Fig. 2B), respectively. At these FICI values, 99 % and 96 % biofilm inhibition was achieved against *C. albicans* and *C. dubliniensis,* respectively. Table 3 indicates the MIC, FIC and FICI of both AmB and C18:4 n-3 against *C. albicans* and *C. dubliniensis* and the interaction of the antifungal compounds was determined to be synergistic.

It can be noted that against *C. albicans*, 31.25 µM of C18:4 n-3 and 2.5 mg/L of AmB will be sufficient in inhibiting biofilm formation. Interestingly, against *C. dubliniensis* higher dosages of C18:4 n-3 (250 µM) and lower dosages of AmB (1.25 mg/L) were observed to provide similar inhibition. These results showed that lower dosages to the initially determined MICs would be sufficient in achieving similar levels of inhibition. Even though combinational usage of these antifungal compounds resulted in lower

dosages able to inhibit biofilm formation, the cytotoxicity of each compound should be taken into account. Cytotoxicity concentrations of AmB ranged between 100-1000 mg/L when AmB was used to inhibit fungal biofilms [Butler, 1966; Sabra & Branch, 1990]. This is significantly higher than the concentrations used in this study. Furthermore, the antifungal fatty acid, C18:4 n-3, was shown to be toxic at concentrations of 120 µmol/L (120 µM) against a mammalian model (mouse) [Hsueh *et al.*, 2011] and it will be important to consider this because of the impact it may have on humans. Therefore, to avoid risks of cytotoxicity, lower dosages of C18:4 n-3 may be required if used in combination with AmB especially against *C. dubliniensis.*



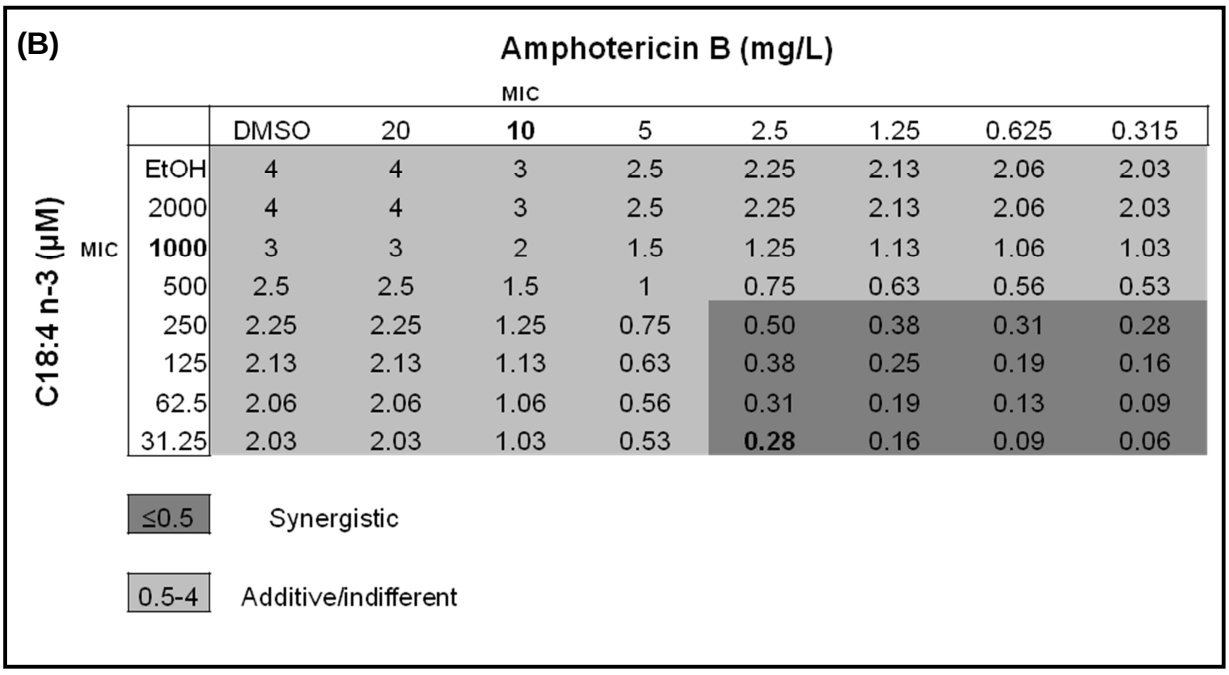
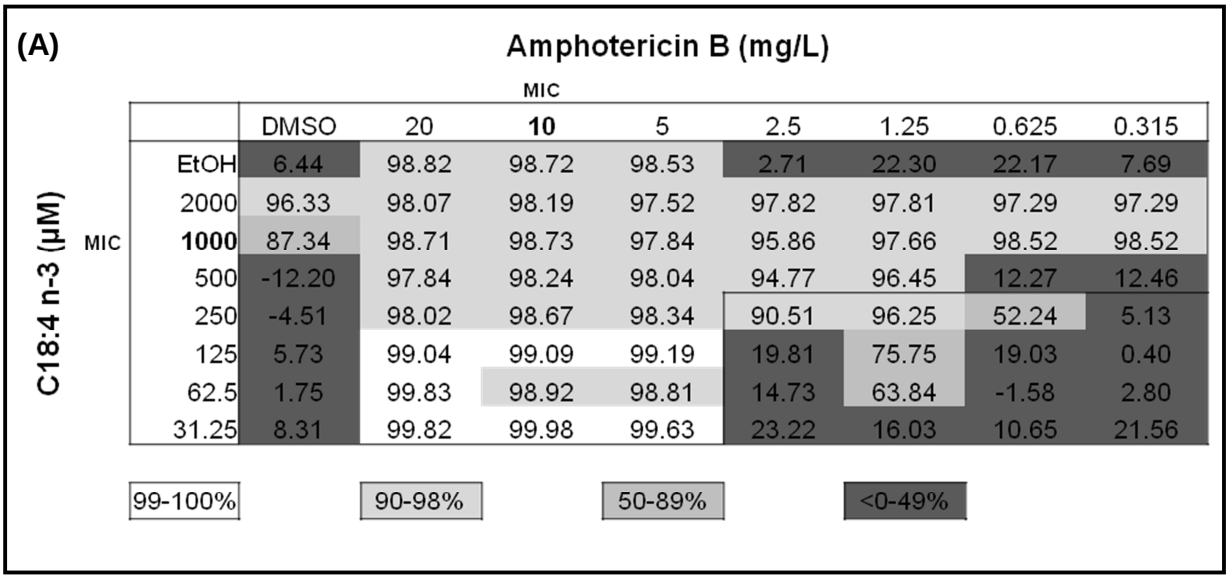


Fig. 1: Effect of combination of AmB and C18:4 n-3 on *C. albicans* biofilms. (1A) Average percentage inhibition of reduction of XTT. (1B) Fractional inhibitory concentration index (FICI) of AmB and C18:4 n-3. The MIC's for both AmB and C18:4 n-3 are indicated. DMSO; Dimethyl sulfoxide, EtOH; Ethanol.



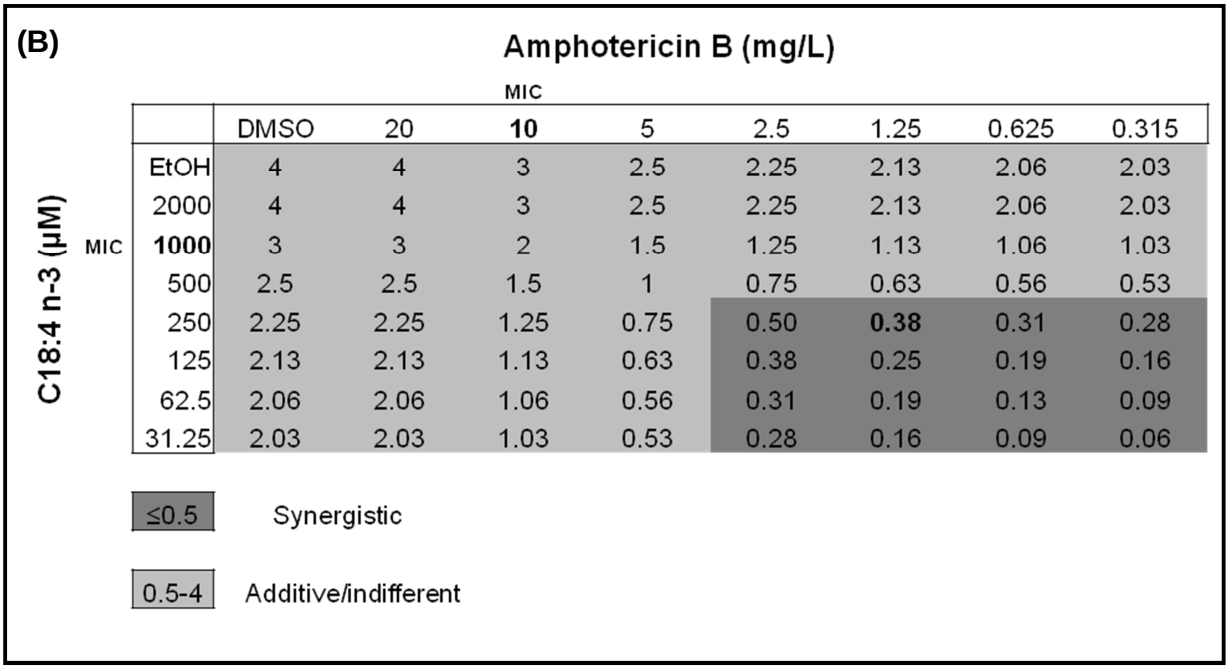


Fig. 2: Effect of combination of AmB and C18:4 n-3 on *C. dubliniensis* biofilms. (1A) Average percentage inhibition of reduction of XTT. (1B) Fractional inhibitory concentration index (FICI) of AmB and C18:4 n-3. The MIC's for both AmB and C18:4 n-3 are indicated. DMSO; Dimethyl sulfoxide, EtOH; Ethanol.

Table 3: The fractional inhibitory concentration index (FICI) showing the synergistic effect of two antifungal compounds, AmB and C18:4 n-3.

	C. albicans CBS 562T			*C. dubliniensis* NRRL Y-17841T		
	MIC	FIC	FICI	MIC	FIC	FICI
C18:4 n-3 (μM)	31.25	0.0313	0.28	250	0.25	0.38
Amphotericin B (mg/L)	2.5	0.25		1.25	0.125	

4.5. Conclusions

Fatty acids have long been known to have antifungal properties and are proposed to target fungal membranes [Avis & Belanger, 2001]. They increase membrane fluidity, leading to membrane disruption and eventually leakage of cellular material and cell death [Avis, 2007]. During our studies we found that C18:4 n-3 has antifungal properties against *C. albicans* and *C. dubliniensis* leading to inhibition of biofilm formation. The antifungal compound, AmB is also known to target fungal membranes by binding to membrane ergosterol, leading to pore formation, leakage of intracellular contents and eventually cell death. Other studies have demonstrated the induction of apoptosis as a possible antifungal mechanism of AmB. Amphotericin B and C18:4 n-3 display almost similar mode of action and therefore present an ideal model for measurement of the synergistic interaction between the two antifungal compounds. However, both AmB and C18:4 n-3 can be toxic when used in high concentrations; therefore one should always strive to use the lowest effective dose.

The results from our studies showed that the AmB and C18:4 n-3 had a synergistic interaction at low concentrations, leading to inhibition of biofilm formation by *C. albicans* and *C. dubliniensis*. Interestingly, against *C. albicans*, higher concentrations of AmB

were needed to achieve inhibition while against *C. dubliniensis* higher concentrations of C18:4 n-3 were needed. This might be due to the differences in the cell wall of *C. albicans* and *C. dubliniensis* which allows them to react differently to compounds used during supplementation. Future studies should investigate how the cell walls of the two species are regulated during antifungal supplementation.

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GENERAL DISCUSSION

In recent years biofilm formation has been implicated in the pathogenesis of several fungal infections. The development of resistance of biofilms towards antifungal supplementation has necessitated the search for possible novel antifungal compounds that can be used to combat these fungal infections. Studies have also focused on combinational therapy of antifungal compounds currently in use with some natural compounds (such as fatty acids). Literature has shown the ubiquitous fatty acids to induce antimicrobial activity depending on the degree of saturation and chain length. The long chain polyunsaturated fatty acid has drawn attention as antifungal compounds due to their relatively high peroxidisability, leading to the induction of lipid peroxidation. The rationale of this study was then to screen and identify possible antifungal fatty acids that can possibly be used alone or in synergism with amphotericin B to combat biofilm formation by both *C. albicans* and *C. dubliniensis*. The study aimed also at finding a possible mechanism which the antifungal fatty acids induced their activity. During the screening of fatty acids, measuring mitochondrial metabolic activity, it was evident that certain fatty acids (saturated, monounsaturated and polyunsaturated) were able to inhibit biofilm formation between *C. albicans* and *C. dubliniensis* at varying percentages. The error margin of percentage of biofilms inhibited with some of the fatty acids was relatively large between the two species. The amount of biofilm biomass inhibited during fatty acid supplementation was measured and a linear correlation with the mitochondrial metabolic activity inhibited was evaluated. Polyunsaturated fatty acids (PUFAs) significantly inhibited both biofilm biomass production and mitochondrial metabolic activity against both *C. albicans* and *C. dubliniensis* and were selected for further analysis. Interestingly, these PUFAs also affected cellular morphology of biofilms of

both *C. albicans* and *C. dubliniensis* resulting in formation of irregular cell wall structures. Formation of these irregular structures was proposed to be due to increased oxidative stress. These observations further opened investigations to evaluate on the possible mode of action of these PUFAs.

When these PUFAs were supplemented in the growth media they were incorporated into the mitochondrial membrane phospholipids. Polyunsaturated fatty acids were taken up depending on their chain length with very long PUFAs slightly incorporated into the membrane phospholids. They increased the unsaturation index of the membrane phospholipids leading to increases in lipid peroxidation, an indication of oxidative stress. The membrane-PUFAs interaction in biofilms of *C. albicans* and *C.dubliniensis* indicated the efficiency of antifungal PUFAs. Because the membrane composition of both *C. albicans* and *C. dubliniensis* differ, different PUFAs interact differently and induced their antifungal activity at varying rates. During our study we were able to report that PUFAs which inhibited biofilm formation, initiated apoptosis through the induction of oxidative stress.

The findings of this study will have significant contribution in the medical industry in combating fungal infections. The study further revealed that antifungal PUFAs can be used in combination with lowered dosages of amphotericin B. This will be advantageous in prolonging their usages and offering a broader spectrum of antifungal compounds available. Although we were able to show the importance of PUFAs as alternative antifungal compounds, further studies into the level of toxicity of this antifungal PUFAs for human utilization still need to be performed.

SUMMARY AND KEYWORDS

Candida albicans and *C. dubliniensis* are commensals of the gastrointestinal and genitourinary tract in healthy individuals. However, in diseased individuals they can cause superficial infections to deep seated mycoses. Both species form mycelial networks called biofilms, and formation of biofilms results in increased resistance towards antifungal compounds currently in use. Therefore, there is a need for alternative antifungal compounds such as fatty acids. Research has shown that supplementation of growth medium with polyunsaturated fatty acids (PUFAs), increased the unsaturation index and made cells susceptible to lipid peroxidation and cell death. During this study this phenomenon was evaluated on biofilms of *C. albicans* and *C. dubliniensis* using selected PUFAs. Due to differences in the carbon chain length and saturation of fatty acids, they interact differently with the cell membrane and will have different peroxidisability values. The results from the study showed C18:4 n-3 and C20:5 n-3 were taken in by the cell and resulted in increased unsaturation index. The results further indicated oxidative stress-induced apoptosis following supplementation with C18:4 n-3 and C20:5 n-3 in biofilms of both *C. albicans* and *C. dubliniensis*. The induction of apoptosis following supplementation by C18:4 n-3 and C20:5 n-3 was confirmed by mitochondrial membrane potential assay, Annexin V-FITC staining, TUNEL assay and DAPI staining. The use of C18:4 n-3 in synergism with amphotericin B resulted in decreased dosage of the antifungal compound needed to inhibit biofilms of *C. albicans* and *C. dubliniensis*.

Keywords:

Amphotericin B, Antifungal, Apoptosis, Biofilms, *Candida albicans*, *Candida dubliniensis*, Lipid peroxidation, Oxidative stress, Polyunsaturated fatty acids, Reactive oxygen species, Synergistic interaction

OPSOMMING EN SLEUTELWOORDE

Candida albicans en *C. dubliniensis* is kommensale van die gastro-intestinale en genitor-urinêre weg van gesonde individue. In siek individue kan hulle egter oppervlakkige tot sistemiese mikoses veroorsaak. Beide spesies vorm misiliêre netwerke wat biofilms genome word, en biofilmvorming veroorsaak verhoogde weerstand teen antifungale middels wat tans in gebruik is. Dus is daar ‘n behoefte aan alternatiewe antifungale middels soos vetsure. Navorsing het getoon dat byvoeging van polionversadigde vetsure (PUFAs) tot groeimedia, die onversadigingsindeks verhoog en selle vatbaar maak vir lipiedperoksidasie en dood. Hierdie verskynsel is gedurende hierdie studie geëvalueer op biofilms van *C. albicans* en *C. dubliniensis* met sekere PUFAs. As gevolg van die verskille in koolstofkettinglengte en versadiging van vetsure verskil die interaksies wat hulle met selmembrane het en sal hulle verskillende vlakke van peroksideerbaarheid hê. Die resultate van hierdie studie het aangetoon dat C18:4 n-3 en C20:5 n-3 deur die selle opgeneem is en ‘n toename in onversadigingsindeks veroorsaak het. Verder het die reulate getoon dat behandeling van beide *C. albicans* en *C. dubliniensis* biofilms met C18:4 n-3 en C20:5 n-3 gelei het tot oksidatiewe stress veroorsaakde apoptose. Die induksie van apoptose na behandeling met C18:4 n-3 en C20:5 n-3 is bevestig met ‘n mitochondriale membraanpotensiaaltoets, Annexin V-FITC kleuring, TUNEL toets en DAPI kleuring. Die gebruik van C18:4 n-3 in sinergisme met amfoterisien B het gelei tot ‘n verlaagde dosis van die antifungale middel wat nodig was om biofilms van *C. albicans* en *C. dubliniensis* te inhibeer.

Sleutelwoorde:

Amfoterisien B, Antifungaal, Apoptose, Biofilms, *Candida albicans*, *Candida dubliniensis*, Lipiedperoksidasie, Oksidatiewe stress, Polionversadigde vetsure, Reaktiewe suurstofspesies, Sinergistiese interaksie