The Effect of Arachidonic acid on Lipid Metabolism and Biofilm Formation of Two Closely Related *Candida* species

by

Ruan Ells

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

Supervisor: Dr C.H. Pohl
Co-supervisor: Prof J.L.F. Kock

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This dissertation is dedicated to the following people: My father, *R. Ells*; mother, *L. Ells*; sister, *M. Ells*; brother, *J. Ells* and to my love, *L. Krüger*.

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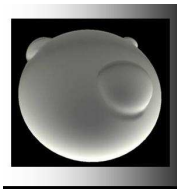
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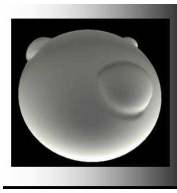
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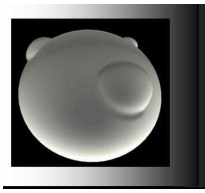
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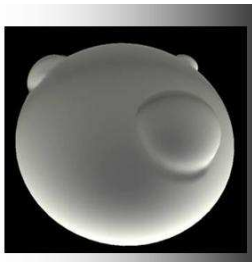
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CHAPTER 1

Literature review

1.1) Motivation

Candida albicans and *C. dubliniensis* are closely related species and important in nosocomial infections (Sullivan *et al*., 1995). They are increasing as opportunistic pathogens in HIV infected and AIDS patients, causing candidemia and candidiasis worldwide. Immunocompromised individuals are increasing due to the increasing frequency of HIV infected individuals, those using antimicrobial drugs, receiving cancer chemotherapy and immunosuppressive agents after organ transplantation (Coleman *et al*., 1997). These individuals are more susceptible to infections by these yeasts. Biofilms are believed to be the causative agent of infections and drug resistance in *Candida* species, because of their difficulty to remove (Jabra-Rizk *et al*., 2004). As a result, biofilms are increasing as an important health problem for patients with microbial infections.

Today, two classes of membrane active antifungals are used to treat *Candida* infections, the polyenes and the azole drugs (Al-Mohsen & Hughes, 1998). However, *Candida* species have gained the ability to develop resistance towards these antifungals and it is known that some of these antifungals (amphotericin B) have a high toxicity towards the host (Al-Fattani & Douglas, 2004; Al-Mohsen & Hughes, 1998; Graybill, 2000). Arachidonic acid can be incorporated into yeast cell membranes thereby influencing the saturation. Strikingly, it was found that unsaturation plays a role in the susceptibility towards antifungals (Hąc-Wydro *et al*., 2007; Yamaguchi, 1977; Yamaguchi & Iwata, 1979).

It is known that several pathogenic fungi, including *C. albicans*, utilise the arachidonic acid cascade or are capable of producing arachidonic acid metabolites, eicosanoids, either as a mechanism of virulence or as morphogenic factors (Noverr *et al*., 2002). It is known that these eicosanoids, produced by *C. albicans*, have the ability to stimulate inflammatory responses in the host (Erb-Downward & Huffnagle, 2006; Noverr *et al*., 2002). Lipids also contribute considerably to fungal pathogenesis and a better understanding of the metabolism of lipids, in these fungal pathogens, might help to develop more useful approaches for antifungal therapies (Mishra *et al*., 1992).

1.2) Introduction

Many fungi have the ability to cause human diseases, mycoses, as either true pathogens or opportunistic pathogens (Xiaogang *et al*., 2003). Important fungal pathogens include the species belonging to the genus *Candida*, classified as yeasts with ascomycetous affinity (Kurtzman & Fell, 1998). Some of these species exist as commensals of mucosal membranes in most healthy individuals and other warm-blooded animals, where they grow without causing any damage (Ramage *et al*., 2001a; Sullivan *et al*., 2004). However, in some cases due to a change in the environment, they can become pathogenic. *Candida albicans* is an example of such an opportunistic pathogen, causing infections ranging from mild to life threatening.

These infections can be grouped into two categories. The first is superficial mucocutaneous infections that are usually found in the exposed and moist parts of the body, for example in the oral (involving the buccal mucosa, palate and tongue), gastrointestinal and genital areas (vaginitis) (Ruiz-Sanchez *et al*., 2002). This is known as candidosis or candidiasis. In immunocompromised patients, especially those with HIV and AIDS, these mucocutaneous infections can become systemic invasive infections, which involve the spread via the blood stream to the major organs, causing endocarditis, pyelonephritis, esophagitis, meningitis and disseminated candidiasis (Edmond *et al*., 1999; Launay *et al*., 1998). These are the second group of infections and are known as candidemia and are associated with a significant morbidity and mortality.

These serious infections are increasing due to an increase in immunocompromised patients, as a result of the increasing frequency of HIV infection, the use of antimicrobial drugs, cancer chemotherapy and the use of immunosuppressive agents after organ transplantation as well as the increased use of implanted medical devices, such as indwelling central venous catheters (Xiaogang *et al*., 2003). It was found that 80 % of yeasts isolated from black HIV positive South Africans represent *C. albicans*, in contrast to the situation in healthy black and white individuals, in which 58 % and 67 % of the yeast isolates represent *C. albicans* respectively (Blignaut *et al*., 2002).

Until recently *C. albicans* was considered the most important opportunistic pathogen in this genus. However, other non-*albicans Candida* species, such as *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* have also emerged as causative agents of infections (Sullivan *et al*., 2004). *Candida glabrata* has been implicated in superficial and systemic candidiasis in immunocompromised individuals (Fidel *et al*., 1999). However, it is difficult to determine the incidence of *C. glabrata* in these infections, since this species is often isolated as a mixed culture with *C. albicans*. In a study by Fidel *et al*. (1999), it was found that *C. glabrata* was responsible for 20 % of urinary tract infections that primarily occurred in elderly hospitalised, incapacitated, and catheterised patients who have recently received antimicrobial agents. *Candida krusei*, also an opportunistic pathogen, is also primarily associated with urinary tract infections in immunocompromised patients and a major cause of fungal vaginitis, especially in elderly women (Singh *et al*., 2002). A strain of *C. parapsilosis* has been isolated from patients with denture stomatitis and is also an important human fungal pathogen, because of its ability to form biofilms and resultant increased resistance to antifungals (Kuhn *et al*., 2002).

Another non-*albicans Candida* species found to be an emerging opportunistic pathogen is *C. dubliniensis*. Sullivan *et al*. (1995) initially isolated *C. dubliniensis* from AIDS patients in Dublin, Ireland. Because of especially phenotypic similarity between *C. dubliniensis* and *C. albicans* (i.e. germ tube and chlamydospore production as well as biofilm formation), *C. dubliniensis* was previously misidentified as *C. albicans* (Ramage *et al*., 2001b). These similarities still make it difficult to quickly differentiate between the two species, especially in clinical samples. Identification of isolates to the species level is required to help in the selection of the antifungal drug to be used. Therefore different methods have been developed to differentiate between the phenotypic characteristics of these two species. These include morphology on Pal's agar (sunflower seed agar) (Mosaid *et al*., 2003), growth at 42-45 ℃ (Pinjon *et al*., 1998), fluorescence on methyl blue-Sabouraud agar (Yücesoy *et al*., 2001) and their carbohydrate assimilation profiles (Sullivan *et al*., 1995). Although *C. dubliniensis* was originally associated with oral candidiasis in HIV infected patients (15-30 %) worldwide, it is now emerging as a cause of superficial and systemic disease in HIV negative individuals with prevalence

rates below 5 % (Gutierrez *et al*., 2002; Mosaid *et al*., 2001; Sullivan & Coleman, 1998). *Candida dubliniensis* has also been isolated from faeces, urine, wounds, vaginal swabs and respiratory tract specimens of non-HIV infected patients (Gee *et al*., 2002; Jabra-Rizk *et al*., 2000). In South Africa, Blignaut *et al*. (2003) found that colonisation by *C. dubliniensis* is more prevalent in healthy white individuals (16 %) compared to healthy black individuals (0 %). This was also found true in HIV positive individuals with 9 % colonisation in infected white individuals compared to the 1.5 % in infected black individuals. They explained that these differences might be based on cultural (habitat and diet) or racial differences. It was also found that *C. dubliniensis* isolates are susceptible to the antifungals currently in use (i.e. amphotericin B), however they have the ability to rapidly develop resistance to some azole drugs, such as fluconazole, upon contact *in vitro* (Sullivan & Coleman, 1998).

More than ten years after the description of *C. dubliniensis* the incidence of this organism and its relatedness to *C. albicans* is still not completely understood. However, it is known that *C. albicans* is more successful as a pathogen (Sullivan *et al*., 2005). This was indicated by the reduced virulence of *C. dubliniensis* in a comparative study using a mouse model of systemic infection (Sullivan *et al*., 2004).

From this introduction, it is clear that *C. albicans* and *C. dubliniensis* are important opportunistic fungal pathogens and although they are genetically distinct species, they share many phenotypic characteristics that make their identification, in especially clinical settings, difficult. In addition, little is known regarding the differences in virulence factors between these species. Therefore, this review will focus on the similarities and differences between *C. albicans* and *C. dubliniensis* based on their phenotypic and genotypic characteristics, virulence factors, drug resistance and their ability to produce eicosanoids.

1.3) Phenotypic characteristics

Many researchers isolated *Candida* species capable of producing germ tubes and chlamydospores from HIV infected patients in the early 1990s (Gutierrez *et al*., 2002). These characteristics were considered typical of *C. albicans* isolates and were used for identification. However, later it was realised that some of these isolates differ genetically and in their carbohydrate assimilation profiles from the known *C. albicans* strains. This led to the description of a new species, *C. dubliniensis*, by Sullivan *et al*. (1995). Confirmation studies of two yeast culture collections indicated that about 2 % of *C. dubliniensis* was incorrectly identified as *C. albicans* and in another study of oral yeasts isolated from HIV infected individuals, 16.5 % of *C. dubliniensis* was wrongly identified as *C. albicans* (Coleman *et al*., 1997). Today *C. dubliniensis* is one of the most frequently encountered species together with *C. albicans* in clinical candidiasis (Redding *et al*., 2001).

The most valuable and accurate method of discriminating between these two organisms is by molecular techniques, which will be discussed later, but these methods are expensive, time consuming, not available in every laboratory and not suitable for large numbers of samples (Pinjon *et al*., 1998). This illustrates the need for phenotype-based tests which are accurate, reliable, inexpensive and rapid. Some of these tests, that will be discussed here, include the determination of colony colour on CHROMagar™ Candida plates, growth at 45 ℃ and the carbohydrate assimilation profile (Mosai d *et al*., 2003).

1.3.1) Characteristics on differential media

In designing an isolation and identification medium for clinically important yeasts, several criteria should be taken into consideration. The media should allow the growth of only yeast and not bacteria; it should be able to differentiate between different yeast species found in clinical samples and make it possible to recognise specimens that contain mixtures of yeast species (Odds & Bernaerts, 1994). One medium that satisfies this requirement is CHROMagar™ Candida which uses a chromogenic β-glucosaminidase substrate to identify yeast directly on primary plates (Cooke *et al*., 2002; Pfaller *et al*., 1996). Experiments performed to test this medium indicated that it inhibits the growth of bacteria, allows the growth of clinically isolated yeasts

and has an extremely high discriminating power among mixed yeast species (Odds & Bernaerts, 1994). Differentiation on CHROMagar™ Candida is based on colour differences between different *Candida* species after growth at 37 ℃ for 48 h because of the presence of species-specific enzymes. *Candida albicans* produce β-N-acetylgalactosaminidase which interacts with the chromophore (chromogenic hexosaminidase substrate) incorporated into the agar. However, it is still unknown which enzymes are responsible for the different colony colour formation in the other *Candida* species. *Candida albicans* colonies appear light blue-green, *C. krusei* colonies appear pale pink, *C. tropicalis* colonies appear bluish purple and *C. glabrata* colonies appear pink (Fig. 1).



Fig. 1. Four different *Candida* species incubated for 48 h at 37 ℃ on CHROMagar™ Candida. The species can be distinguished by their colony appearances with two *C. glabrata* colonies being pink, two *C. tropicalis* colonies are bluish-purple four *C. albicans* colonies are green and the two large rough, pale pink, colonies are *C. krusei* (Odds & Bernaerts, 1994).

These experiments were performed before the identification of *C. dubliniensis*, but this medium was also found to be very useful and reliable in identification of *C. dubliniensis* isolates (Sullivan *et al*., 1999). *Candida dubliniensis* isolates form dark green colonies after incubation at 37 ℃ for 48 h, with the colour being more prominent after 72 h of incubation. However, it should be noted that *C. dubliniensis* might lose its ability to form this distinct dark green colour after subculturing and storage (Pincus *et al*., 1999; Schoofs *et al*., 1997). The reason for this colour instability might be due to the higher frequency of

phenotypic switching observed for *C. dubliniensis* isolates compared to *C. albicans* isolates (Hannula *et al*., 2000; Tintelnot *et al*., 2000).

Mosaid *et al*. (2003) used Pal's agar to differentiate between these two species. Pal's agar contains sunflower (*Helianthus annuus*) seed extract and was initially developed for the identification of *Cryptococcus neoformans*. When *C. albicans* and *C. dubliniensis* were grown on this medium it was found that all the isolates formed smooth creamy-grey colonies, after 48-72 h incubation. However, *C. dubliniensis* isolates had hyphal fringes, grew as rough colonies, produced chlamydospores and *C. albicans* did not (Fig. 2).

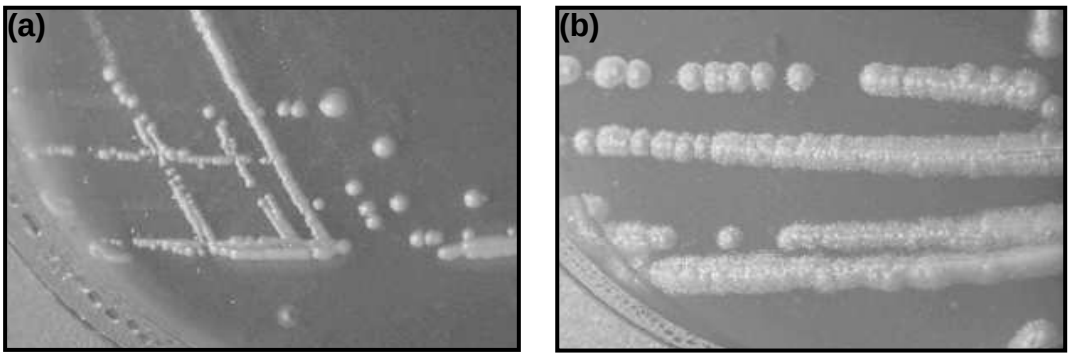


Fig. 2. *Candida albicans* and *C. dubliniensis* colonies after 72 h of incubation on Pal's medium at 30 ℃. (a) Smooth colonies of *C. albicans*. (b) *C. dubliniensis* rough colonies with a hyphal fringe (Mosaid *et al*., 2003).

They also found that incubation at 30 ℃ rather than 37 ℃ give a better differentiation between these organisms. However, Sahand *et al*. (2005) later indicated that the other *Candida* species had the same colony morphology on this media as *C. albicans* and *C. dubliniensis*, making differentiation between species in mixed cultures difficult. Sahand *et al*. (2005) combined CHROMagar™ Candida with Pal's agar, by mixing equal volumes of both media, and it was possible to identify *C. dubliniensis* in mixed cultures including *C. albicans*, *C. glabrata*, *C. krusei* and/or *C. tropicalis* (Fig. 3).

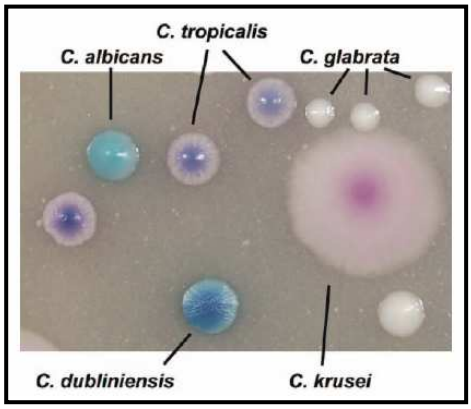


Fig. 3. *Candida* species incubated for 48 h at 37 ℃ on CHROMagar™ Candida medium supplemented with Pal's agar forms distinctive colony colours for discrimination (Sahand *et al*., 2005).

Another medium, on which the same effect of chlamydospores and hyphal fringe production by *C. dubliniensis* but not *C. albicans* was observed, and which is also frequently used for rapid identification of these species, is tobacco agar (Khan *et al*., 2004). Hundred percent differentiation between 54 *C. albicans* and 30 *C. dubliniensis* isolates were possible at 30 ℃. In contrast to this Kumar & Menon (2005) found that tobacco agar is a good medium only for the differentiation of *C. albicans* and *C. dubliniensis* from other *Candida* species. They found that 96 % of *C. albicans* strains tested and a 100 % of *C. dubliniensis* strains tested produced chlamydospores on tobacco agar after incubation and none of the other *Candida* strains tested.

Another medium developed by Kumar *et al*. (2006) for the differentiation of *C. albicans* and *C. dubliniensis* from other *Candida* species is mustard agar. This medium allowed the growth of 60 *Candida* isolates, including *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. tropicalis* as well as *C. albicans* and *C. dubliniensis*. However, only *C. albicans* and *C. dubliniensis* had the ability to produce chlamydospores but with no difference in colony morphology between them. This medium has proved useful for the identification of these two species in a mixed clinical sample of *Candida* species.

It is known that *C. dubliniensis* tends to form pairs or triplets of chlamydospores on the ends of short-branched hyphae, and in the case of *C. albicans*, a single chlamydospore on the tip of a suspensor cell is formed

(Sullivan *et al*., 1995). However, care should be taken when using chlamydospore production to differentiate between the species. In contrast to Sullivan's work, Ellepola *et al*. (2003) demonstrated on corn meal-Tween agar that the differentiation between these two species based on chlamydospore formation is not reliable, since *C. dubliniensis* showed no constant pattern in chlamydospore formation. In Fig. 4(a), a *C. dubliniensis* strain forms triplets or pairs of chlamydospores on short branched hyphae but in Fig. 4(c), a *C. dubliniensis* strain forms single terminal chlamydospores similar to a *C. albicans* strain (Fig. 4b).

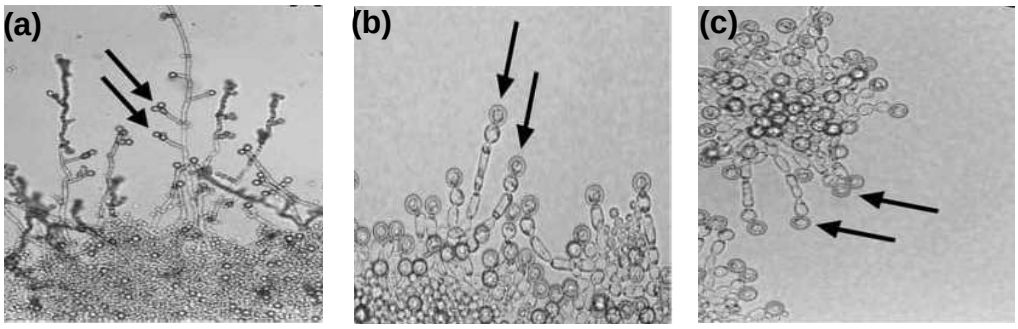


Fig. 4. Morphology of *Candida albicans* and *C. dubliniensis* chlamydospores on corn meal-Tween agar. (a) *C. dubliniensis* strain with triplets or pairs of chlamydospores. (b) *C. albicans* strain forming a single terminal chlamydospore. (c) *C. dubliniensis* strain forming single terminal chlamydospores. Arrows indicate chlamydospores (Ellepola *et al*., 2003).

Methyl blue-Sabouraud agar is also reported as useful for identification (Schoofs *et al*., 1997). Differentiation using this media is based on the ability of *C. albicans* colonies to fluoresce under Wood's light (long wave length UV) and the inability of *C. dubliniensis* to do so. The exact reaction between the methyl blue-Sabouraud dye and *C. albicans* is still unknown, but there might be a reaction with specific cell wall polysaccharides that produces the fluorescent metabolite (Yücesoy *et al*., 2001). However, this medium also has its disadvantages. *Candida albicans* isolates can lose its fluorescence and *C. dubliniensis* can become fluorescent after subculturing and storage of the isolate (Schoofs *et al*., 1997; Sullivan & Coleman, 1998). This indicates that this medium is only useful after initial isolation of a *Candida* culture.

1.3.2) Growth at elevated temperatures

It was found by Sullivan *et al*. (1995) that *C. dubliniensis* can grow well at 30 ℃ and 37 ℃ producing creamy white colonies on sol id media, just as *C. albicans*. However, differing from *C. albicans*, it grew poorly or was unable to grow at 42 ℃ on Sabouraud dextrose agar or potato dextrose agar (Fig. 5). However, Pinjon *et al*. (1998) found that *C. albicans* and *C. dubliniensis* could grow to the same extent at 42 ℃ and decided to gro w these two organisms at even higher temperatures of up to 45 ℃ on Emmons' modified Sabouraud glucose agar. Their results indicated that none of the 120 *C. dubliniensis* isolates tested grew at 45 ℃ after 48 h whereas 11 isolates showed partial growth at 42 ℃ after 48 h. In the case of *C. albicans*, all 98 isolates tested grew at 42 ℃ and 97 grew at 45 ℃. These results m ake this method suitable for rapid and easy differentiation between these organisms.

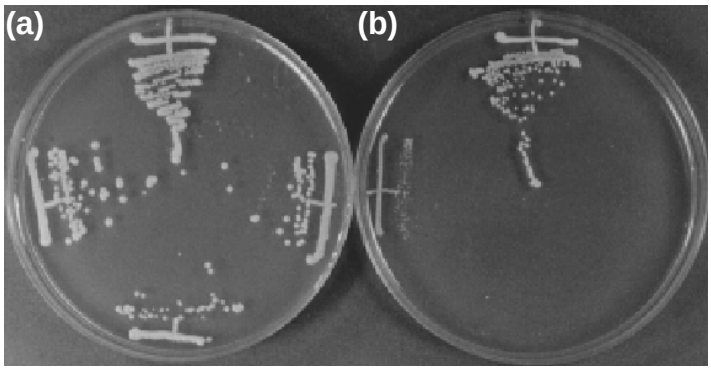


Fig. 5. *Candida albicans* and *C. dubliniensis* oral isolates grown on Potato dextrose agar after 48 h of incubation at (a) 37 ℃ and (b) 42 ℃. Clockwise from the top in each panel are *C. albicans* 132A, *C. dubliniensis* CD57, *C. dubliniensis* CD43 and *C. dubliniensis* CD36 (Sullivan & Coleman, 1998).

1.3.3) Carbohydrate assimilation

Candida isolates have the ability to assimilate a range of carbohydrate compounds as their sole carbon source, and this characteristic has been used for their identification (Sullivan & Coleman, 1998). API ID 32C and 20C AUX systems are some of the commercial kits available which generates a numerical code that can be compared to a list of species in a database. From these systems it was clear that *C. dubliniensis* differs significantly form *C.*

albicans in its carbohydrate assimilation profile (Sullivan *et al*., 1995). Some of these differences include the inability of *C. dubliniensis* to assimilate alpha-methyl-D-glucoside, lactate or xylose. By means of multilocus enzyme electrophoresis it was found that *C. dubliniensis* cannot express β-glucosidase activity which hydrolyses cellobiose to glucose (Boerlin *et al*., 1995). This was also observed when *C. albicans* fluoresced in the presence of methyl-umbelliferyl-labelled β-glucoside in a simple assay designed to discriminate between these two species. However Odds *et al*. (1998) found that 67 of 537 (12.5 %) *C. albicans* isolates were also β-glucosidase negative.

1.3.4) Biofilm formation

A biofilm is an arrangement of microbial cells that have the ability to attach to any surface, particularly in aquatic environments such as industrial water systems as well as medical devices, and are covered in exopolymeric material (EXM) (Jabra-Rizk *et al*., 2004). It is believed that the reasons why organisms form biofilms are to increase the availability of nutrients, for metabolic support, the gaining of new genetic characteristics and to be protected from the environment (Ramage *et al*., 2001a). *Candida* biofilms are highly heterogeneous structures consisting out of a mixture of yeast cells, hyphae, pseudohyphae and EXM. Many forms of candidiasis are associated with biofilm formation, where the cells in the biofilm display characteristics differing from free-living cells. These infections always start with adherence of the cells to a substrate, followed by biofilm formation. Therefore, biofilm formation is considered an important virulence factor of *Candida* species.

Ramage *et al*. (2001b) illustrated that *C. dubliniensis* has the ability to form biofilms on biomaterials *in vitro* and that serum and salivary pellicles can enhance this colonisation. By means of a semiquantitative colorimetric method, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction, the different stages in biofilm formation by *C. dubliniensis* were determined (Fig. 6). These stages were identified as the early adhesion (0-1 h), growth phase (1-6 h), the proliferation phase (6-24 h) and the maturation phase (20-48 h). Chandra *et al*. (2001) identified three different stages during biofilm formation i.e. early phase (0-11 h), intermediate phase (12-30 h) and maturation phase (31-72 h). Using

scanning electron microscopy (SEM), it was clear that mature *C. dubliniensis* biofilms, similar to *C. albicans* biofilms, consisted out of a mixture of yeast cells and filamentous forms embedded in EXM (Fig. 7) (Ramage *et al*. (2001b).

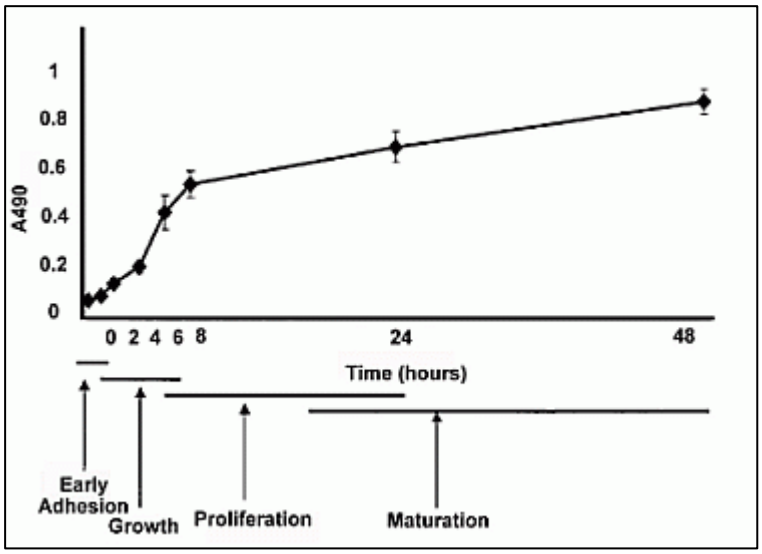


Fig. 6. XTT reduction to determine the kinetics of *Candida dubliniensis* NCPF 3949 biofilm formation. The different phases of biofilm development according to these colorimetric readings are indicated (Ramage *et al*., 2001b).

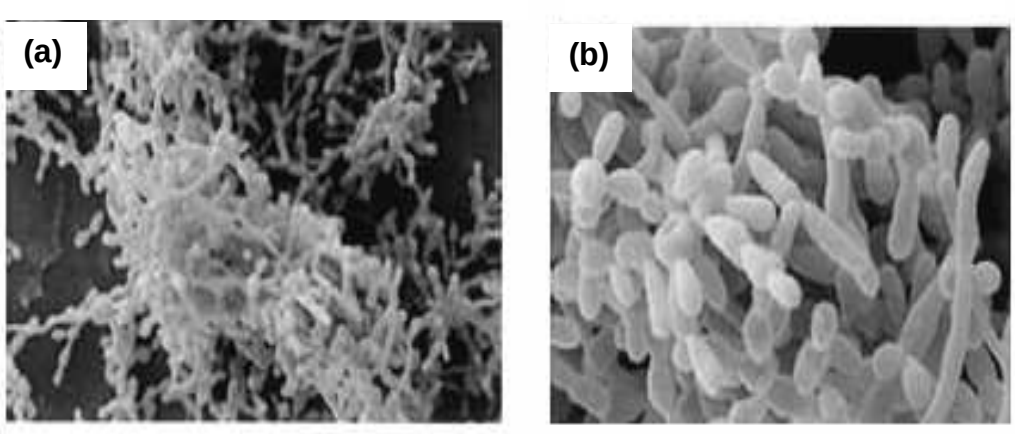


Fig. 7. Scanning electron micrograph (SEM) images of mature (48 h) *Candida dubliniensis* NCPF 3949 biofilms formed on polymethylmethacrylate disks. The same biofilm area is shown at different magnifications. (a) 1000X. (b) 3000X (Ramage *et al*., 2001b).

Biofilm formation of these two species were also compared using crystal violet staining to quantify the total biomass formed, and the amount of active cells in

the biofilm were quantified through XTT reduction (Henriques *et al*., 2006). These authors found that biofilm formation is either strain or species specific. Because of the presence of various cell layers covered in EXM in the biofilm, it was found that an increase in cell number (measured by crystal violet) did not correlate to an increase in metabolic activity (measured by XTT reduction). The metabolic activity decreases because nutrients became limited at the bottom of the biofilm.

Kuhn *et al*. (2003) indicated some limitations of the XTT assay, including the fact that there is not a definite certainty of a relationship between the colorimetric signal and the cell number. Another limitation is that one cannot compare different strains or organisms without standardisation techniques, since the capability of different strains or organisms to metabolise the substrate, differs. The amount of formazan product held back in the cell might also differ between strains and states, such as planktonic and biofilm.

However, Henriques *et al*. (2006) indicated that the metabolic activity (XTT assay) can be correlated to biomass (crystal violet staining) to quantify cell concentration in a biofilm, confirming what Jin *et al*. (2003) found. In their study, Jin *et al*. (2003) compared the biofilm forming ability of *C. albicans* strains from HIV infected and HIV free patients and found a linear relationship between cell activity (measured by XTT) and the cell concentration.

In conclusion, care should be taken in using these rapid identification techniques, since overlap in phenotypic characteristics exist between certain strains of *C. albicans* and *C. dubliniensis*. Thus, if in doubt about the identity of the organism, more precise identification methods such as molecular techniques should be used.

1.4) Genotypic characteristics

As can be seen from the previous discussion, phenotypic characteristics are variable, may lead to incorrect classification of the species and are time consuming (Pinjon *et al*., 1998). A more reliable approach in discriminating between organisms is based on molecular techniques, for example DNA fingerprinting, pulsed field-gel electrophoresis and DNA-DNA hybridization. Some of these techniques and their applications will be discussed here in more detail.

1.4.1) DNA fingerprinting patterns

Candida dubliniensis was initially identified by DNA fingerprinting patterns. It was found that this organism differed from the usual pattern found for *C. albicans* when the *C. albicans*-specific DNA fingerprinting probe, 27A, was used (Sullivan *et al*., 1995). This specific probe gives a fingerprinting pattern of 10-15 strongly hybridizing bands, varying from 500 bp to 20 kb, when the DNA is digested with *Eco*R1, in the case of *C. albicans* (Fig. 8). But *C. dubliniensis* isolates produce only four to seven faint bands that varied between five and 20 kb in size (Fig. 8). Sullivan *et al*. (1995) confirmed results found with the 27A probe, by hybridizing five synthetic oligonucleotide primers $(GGAT)_4$, $(GACA)_4$, $(GATA)_4$, $(GT)_8$ and $(GTG)_5$ with the atypical *C. albicans* isolates (identified as *C. dubliniensis*) as well as with *C. albicans* (132A and CM3) and *C. stellatoidea* (ATCC 11006 and ATCC 20408) strains. Their results again indicated that the atypical strains (i.e. *C. dubliniensis*) had similar profiles, which differed from both *C. albicans* and *C. stellatoidea*.

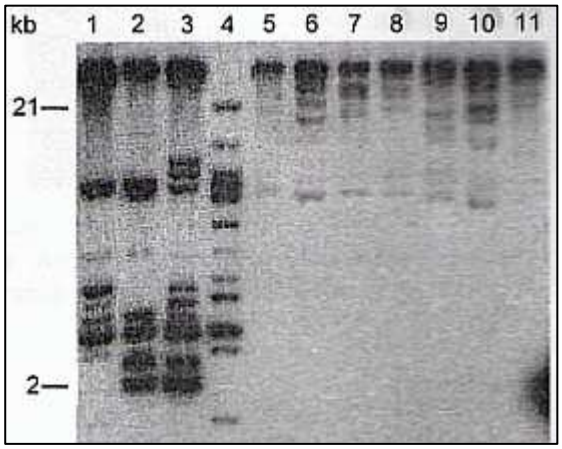


Fig. 8. Southern blot analysis of *Eco*R1 digested total genomic DNA from *Candida albicans* and *C. dubliniensis* isolates probed with *C. albicans*-specific DNA fingerprinting probe, 27A. The fingerprints shown correspond to *C. albicans* (lane 1-2), *C. stellatoidea* (lane 3-4) and *C. dubliniensis* (lane 5-11). The size reference markers are indicated in kb on the left of the figure (Sullivan *et al*., 1995).

By using a broad range of DNA profiling techniques, such as fingerprinting with oligonucleotide primers homologous to eukaryotic microsatellite sequences, pulsed-field gel electrophoresis (PFGE) and specific or randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) analysis, it is clear that there is a considerable difference in the chromosomal arrangement of sequences in each of these species (Bennett *et al*., 1998; Gutierrez *et al*., 2002; Ruhnke *et al*., 1999; Sullivan *et al*., 1995).

Pulsed-field gel electrophoresis was developed by Schwartz *et al*. (1983) to separate much larger pieces of DNA (over 10000 kb in size) compared to continuous (conventional) agarose gel electrophoresis (30-50 kb in size) (Basim & Basim, 2001). It is known that PFGE gives a good resolution and reproducible separation of *Candid*a chromosomes (Jabra-Rizk *et al*., 2000; Lasker *et al*., 1989).

Randomly amplified polymorphic DNA is a technique based on PCR amplification using one or more short oligonucleotide primers to amplify target genomic DNA sequences which then separate species from *C. albicans* according to size by gel electrophoresis, giving a unique banding pattern (Neppelenbroek *et al*., 2006). The closer organisms are related, the more similar the banding patterns would be compared to unrelated organisms. This technique was used in the identification of *C. dubliniensis* as a separate species form *C. albicans* and is still used to differentiate between these two species in clinical samples (Alves *et al*., 2001; Sullivan *et al*., 1995).

1.4.2) Sequence of the internal transcribed spacer regions of ribosomal DNA

For the identification of fungi on the species level as well as for the identification of medically important yeasts, the internal transcribed spacer regions which are highly variable sequences, are used (Fig. 9). (Chen *et al*., 2000a; 2001). Ellepola *et al*. (2003) demonstrated that important *Candida* species could be identified by means of this technique. This technique is based on a PCR assay using universal fungal primers (ITS3 and ITS4) to the internal transcribed spacer 2 (ITS2) regions of ribosomal DNA (rDNA) (Fig. 9). The PCR amplicons are then detected by species-specific DNA probes, which are 5′ end-labeled with digoxigenin, and an all-*Candida* species DNA capture

probe which is 5′ end-labeled with biotin, in an enzyme immunoassay format. In their study they used 22 *C. dubliniensis* and 11 *C. albicans* isolates from different anatomical sites (blood, oral cavity, rectum, vagina) and from different countries (Australia, Belgium, France, Switzerland, USA). *Candida glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* were also included. From their results, it was indicated that the *C. dubliniensis* ITS2 probe was specific for the identification of *C. dubliniensis* DNA and did not cross-react with DNA from any of the other species. The same specificity was observed for the other *Candida* species specific probes.

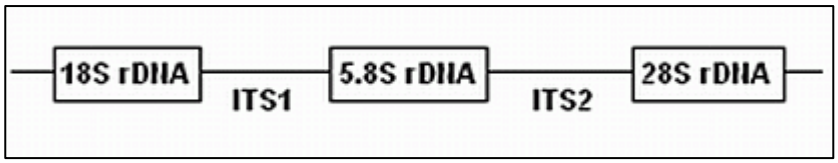


Fig. 9. Diagram of the internal transcribed spacer regions indicating the position of the ITS1 and ITS2 regions on the ribosomal genes (Liguori *et al*., 2007).

1.4.3) Phylogenetic relationships

Although the genotypic characteristics mentioned indicated that the species are distinct, they did not indicate the genetic relatedness between these species. Phylogenetic studies were performed by comparing the nucleotide sequences of the entire small subunit ribosomal RNA (rRNA) gene of different species (Boucher *et al*., 1996; Gilfillan *et al*., 1998). Gilfillan *et al*. (1998) amplified the gene that encodes the small subunit rRNA of the type strain of *C. dubliniensis*, by using specific primers for the conserved sequences (i.e. primers A and B), and compared the sequence obtained to sequences from known yeast species. After PCR amplification and cloning into a vector, a product of 1791 bp was found. After multiple sequence alignments were performed, the sequence was found to differ from the *C. albicans* sequence by 1.4 %. Other yeast sequences that were compared included *C. glabrata*, *C. krusei*, *C. lusitaniae*, *C. tropicalis* and *Saccharomyces cerevisiae*. From these data an evolutionary tree (Fig. 10) was constructed, using the neighbour-joining method of Saitou & Nei (1987). This indicated that *C. dubliniensis* is

phylogenetically distinct from other *Candida* species, including *C. albicans*. These results are comparable to those of Sullivan *et al*. (1995), in their discovery of *C. dubliniensis*. These authors amplified 500 bp from the V3 variable region of the large subunit rRNA genes, from genomic DNA of nine atypical *C. albicans* isolates (i.e. *C. dubliniensis*) which included five Irish, three Australian isolates and strain NCPF 3108. These nucleotide sequences were compared to corresponding nucleotide sequences of *C. albicans* strains 132A and 179B, *C. stellatoidea* strains ATCC 11006 and ATCC 20408, *C. tropicalis*, *C. glabrata*, *C krusei*, *C parapsilosis*, *C. kefyr* and an isolate from *Aspergillus fumigatus*. They found that the *C. albicans* sequences were identical to each other; the same was observed for the sequences of the different *C. stellatoidea* strains. It is also clear that the sequences of *C. albicans* and *C. stellatoidea* strains were almost similar to each other, indicating that these organisms are possibly not separate species. The nine *C. dubliniensis* isolates were all identical to each other, but differed from all the *C. albicans* strains at 14 positions and from all the *C. stellatoidea* strains at 13 positions. All the other non-*albicans Candida* species differed considerably from *C. albicans* sequence data. An evolutionary tree was constructed from these data using the neighbour-joining method of Saitou & Nei (1987) (Fig. 11). These results also indicated that *C. dubliniensis* made up a new taxon within the genus *Candida*, closely related to *C. albicans*.

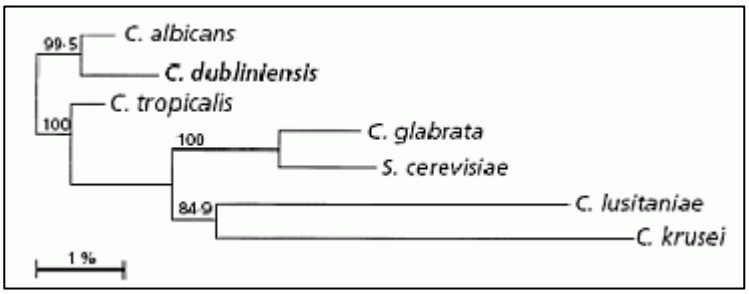


Fig. 10. Unrooted phylogenetic neighbour-joining tree constructed from sequences encoding the small rRNA genes from *Candida dubliniensis* and other *Candida* species. A 1 % difference in nucleotide sequence is represented by the scale bar. Numbers at each node represent the percentage of times the arrangement occurred in 1000 randomly generated trees (Gilfillan *et al*., 1998).

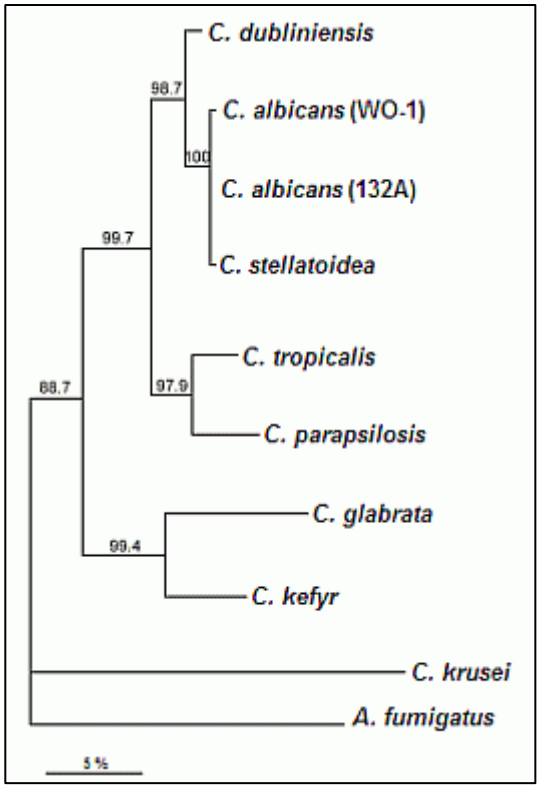


Fig. 11. Unrooted phylogenetic neighbour-joining tree constructed from the alignment of a section of the V3 region from *Candida dubliniensis* and the other species in the tree. A 5 % difference in nucleotide sequence is represented by the scale bar. Numbers at each node represent the percentage of times the arrangement occurred in 1000 randomly generated trees (Sullivan *et al*., 1995).

1.5) Mating of these species

Candida species are classified as anamorphs, with no sexual reproductive cycle present. This was thought to be the case until recently, when mating was observed in *C. albicans* (Bennett & Johnson, 2005). Even before mating was observed a single *MTL* (mating-type like) locus had been identified in *C. albicans* which is normally heterozygous (a/α). To mate, *C. albicans* must undergo *MTL* homozygosis to a/a or α/α. By means of laboratory manipulation, mutations or chromosomal loss, it was possible to create a/a and α/α derivatives, i.e. homozygous strains. It is known that, for *C. albicans* to be able to mate, a phenotypic switch from the stable white phase to the less stable opaque phase has to occur. The change between white hemispherical colonies, known as white (W), and grey flat colonies, known as opaque (O),

influences the size and shape of the cells, its ability to form hyphae, the membrane composition, sensitivity to neutrophils and oxidants, as well as drug susceptibility (Lan *et al*., 2002). The surface of white *C. albicans* cells is smooth and the cells round to slightly ellipsoidal in shape (Pujol *et al*., 2004). Opaque cells are pimpled and elongated. The W/O transition is strain specific with a higher rate in disease-associated isolates. Isolates from invasive infections switch more frequently than isolates from surface infections and have a higher resistance to antifungal therapy. *Candida albicans* homozygous strains (i.e. a/a and α/α) occur naturally between 3 and 7 % in a population, and these strains can undergo W/O switching which is regulated by the *MTL* locus (Bennett & Johnson, 2005). This natural occurrence indicates that *C. albicans* mating can occur in nature.

It is suggested that mating in *C. albicans* is regulated to take place at specific locations in the host environment and at certain suboptimal conditions (Bennett & Johnson, 2005). Interestingly, it was found that opaque-phase cells, which are unstable at 37 ℃ under aerobic co nditions (Dumitru *et al*., 2007), are predominant in cutaneous infections, in contrast with white-phase cells which predominate in systemic infections (Bennett & Johnson, 2005). Therefore, they suggested that mating occurs on the skin, which is aerobic and has a relatively low temperature (31.5 ℃). In addition, Dumitru *et al*. (2007) indicated that opaque-phase cells are more stable at anaerobic conditions at 37 ℃. Therefore the gastrointestinal tract would also be a suitable habitat for mating and colonisation by *C. albicans*.

During mating, opaque α/α cells secrete an α-factor mating pheromone (encoded for by *MF*α gene) and a/a cells, expressing the *STE2* gene (receptor gene), react to this stimulus by growing towards the α/α cells and eventually fuse with them (Bennett *et al*., 2003). Since *C. albicans* is a diploid organism, tetraploid cells are formed during this fusion and because of the absence of a meiotic cycle, chromosomal loss followed by duplication has been found to be the possible mechanism to convert back to the diploid state (Bennett & Johnson, 2003; 2005; Wu *et al*., 2005). This type of mating in *C. albicans* has been termed a parasexual cycle (Forche *et al*., 2008).

In *C. dubliniensis* the same white opaque switching, dependant on *MTL* homozygosis, was observed (Pujol *et al*., 2004). In *C. dubliniensis* mating also

occurs only between a/a and α/α cells and it is also dependent on a W/O switching (Fig. 12). Interestingly, it was found that 33 % of a *C. dubliniensis* population was natural homozygous a/a and α/α strains, which is much higher than those in a *C. albicans* (3-7 %) population (Bennett & Johnson, 2005). This indicates that *C. dubliniensis* has a greater possibility of mating in the environment than *C. albicans*. The morphology of *C. dubliniensis* white and opaque cells are similar to *C. albicans* white and opaque cells, however the opaque cells of *C. dubliniensis* differed by occasionally forming large elongated cells, with hyphal characteristics, but still expressing the opaque phenotype (Fig. 13) (Pujol *et al*., 2004). A difference between mating of these two organisms is that when *C. albicans* opaque a/a and α/α cells mate, they form large, stable clumps in a suspension mixture, but *C. dubliniensis* cells do not clump.

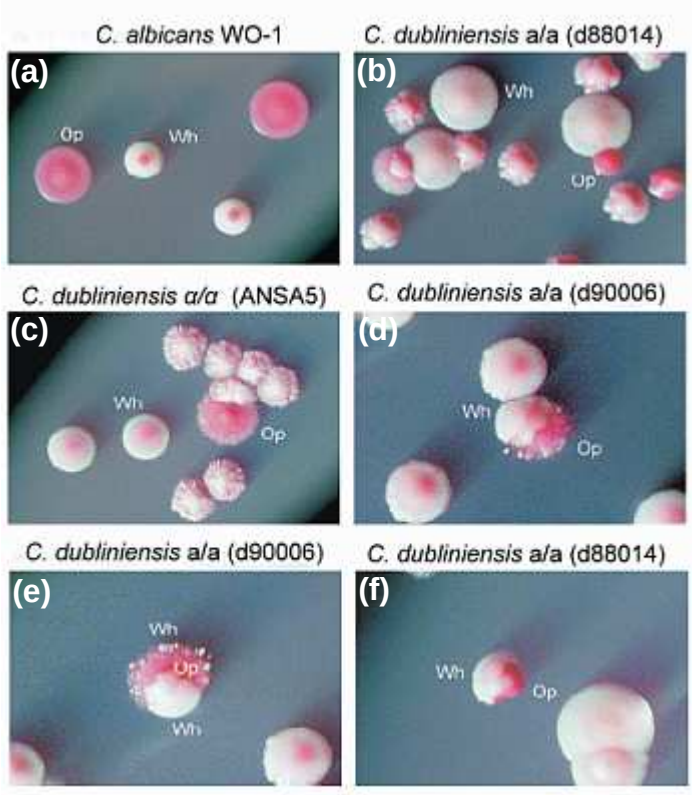


Fig. 12. Strains of *Candida dubliniensis* also have the ability to undergo white opaque switching just as *C. albicans* strains. (a) Switching in *C. albicans* strain WO-1 (α/α). (b to f) Switching of a/a and α/α *C. dubliniensis* strains. Wh, white; Op, opaque (Pujol *et al*., 2004).

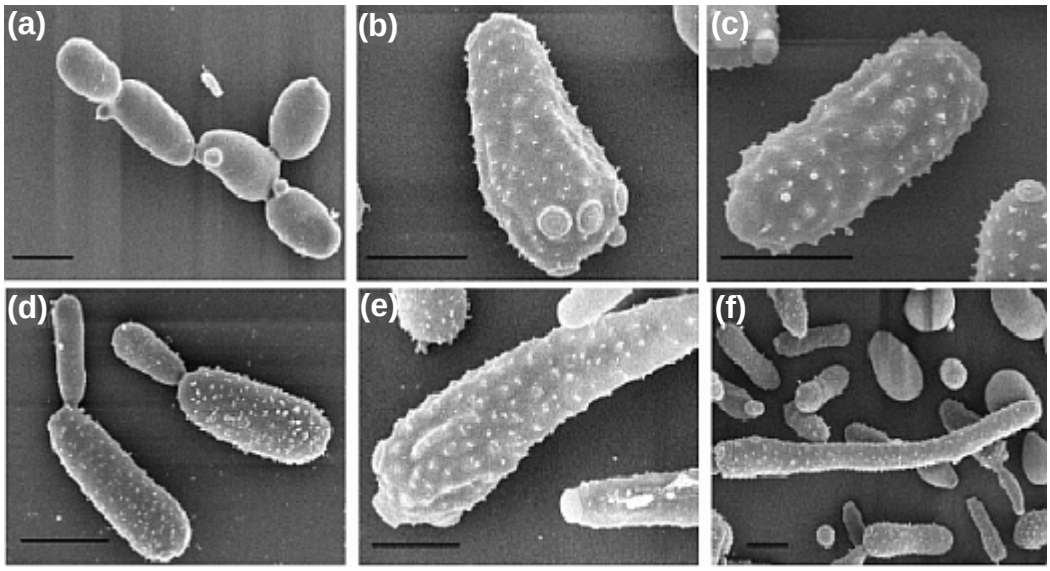


Fig. 13. *Candida dubliniensis* opaque-phase cells have pimples just as *C. albicans* but can become abnormally large and elongated. (a) White cells. (b to d) Opaque cells. (e, f) Large, elongated opaque cells. Note the pimples on opaque cells. Bars, 2 µm (Pujol *et al*., 2004).

Although these two species were proven to be different, Pujol *et al*. (2004) confirmed that these organisms are very similar in their mating patterns and they have the ability to mate with each other. In their examination to determine if these two species can mate, they stained *C. albicans* opaque a/a cells with fluorescein isothiocyanate (FITC)-conjugated ConA (green) and *C. dubliniensis* opaque α/α cells with rhodamine-conjugated ConA (red). After mating the cells were stained with calcofluor white to identify the whole zygote. After 10 h of incubation of an equal number of cells of both species, confocal laser-scanning microscopy revealed the fusants with a light blue colour (Fig. 14). This illustrates a mating-type-dependent fusion between these species. It is known that *C. albicans* uses several of the same cell surfaces and secreted proteins for cell-cell interactions, mating and pathogenesis (Bennett *et al*., 2003). This might illustrate a link between virulence and mating in maybe every pathogenic *Candida* species.

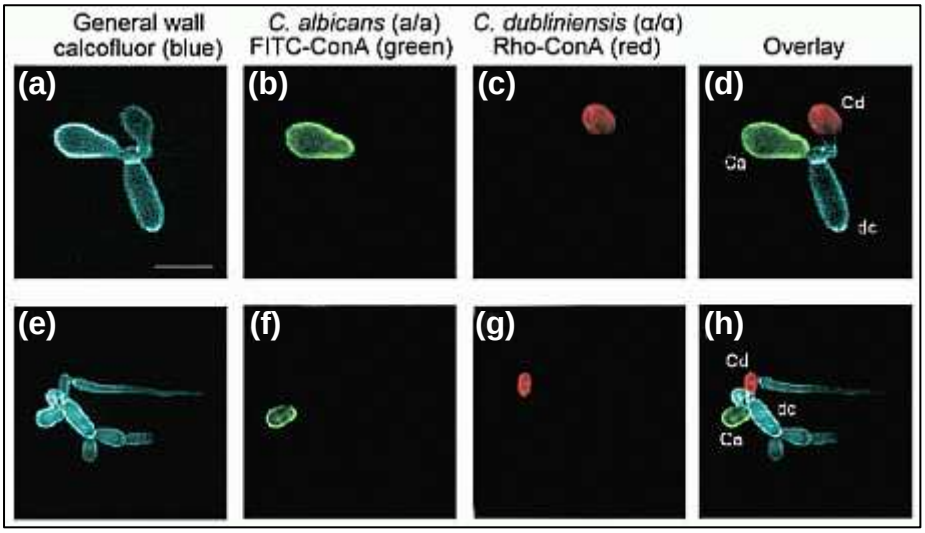


Fig. 14. Interspecies mating between opaque *Candida albicans* a/a cells and opaque *C. dubliniensis* α/α cells. FITC-conjugated ConA (green) stained *C. albicans* strain P37005 (a/a) and rhodamine (Rho)-conjugated ConA (red) stained *C. dubliniensis* strain d126423 (α/α) cells were allowed to mate in suspension cultures for 5 h. (a, e) Fusants stained with calcofluor for cell wall visualisation. (b, f) Selective imaging of FITC-conjugated ConA. (c, g) Selective imaging of rhodamine-conjugated ConA. (d, h) Overlays of calcofluor, FITC-conjugated ConA, and rhodamine-conjugated ConA images (Pujol *et al*., 2004).

1.6) Virulence factors

Virulence is the term specifying the factors that contributes to the ability of an organism to cause a disease (Cutler, 1991). *Candida* is found as commensal organisms that have to undergo certain changes or have special characteristics to become pathogenic (Senet, 1997). For *Candida* to change to a pathogenic state the host defence mechanism has to be overcome and certain virulence factors have to be produced (Gutierrez *et al*., 2002). Factors that aid *Candida* species to be successful as pathogens include cell surface hydrophobicity, adhesion, hydrolytic enzyme production (lipase, phospholipases and secretory aspartyl proteinases) and dimorphism.

1.6.1) Cell surface hydrophobicity

Hydrophobicity of the cell surface may be associated with properties of pathogens that cause them to adhere to epithelial cell surfaces and to plastic medical devices (Gutierrez *et al*., 2002). It therefore plays an important role during adhesion, providing hydrophobic interactions that support the initial bond between the pathogenic cell and the host surface. Cell surface hydrophobicity is a well known phenomenon in *Candida* species. It is still unknown to what extent hydrophobicity influences virulence, but what is known is that hydrophobic cells are more adherent to host and non-living materials, more resistant to phagocytosis and more competent to germinate (Hazen *et al*., 2001; Henriques *et al*., 2004).

A commonly used assay to measure cell surface hydrophobicity is the hydrophobic microsphere assay (CSH assay) using polystyrene microspheres (Hazen & Hazen, 1987). *Candida albicans* displays growth-temperature dependant expression of surface hydrophobicity and is hydrophobic at 23-25 ℃, but not at 37 ℃. In contrast, it was found tha t *C. dubliniensis* has cell surface variations, unlike *C. albicans*, that allow it to be constantly hydrophobic, regardless of the temperature (Jabra-Rizk *et al*., 2001). Henriques *et al*. (2004) measured CSH by investigating the ability of *C. albicans* and *C. dubliniensis* strains to adhere to acrylic and hydroxyapatite surfaces and found that both organisms were hydrophilic at 37 ℃. This difference in results might be due to a drawback of the microsphere based CSH assay, that besides hydrophobic forces, other forces such as electrostatic forces may intervene in the interaction between the microspheres and the cell surface, giving false results. It is clear that hydrophobicity plays a key role in the adhesion process, and that the type of material where adhesion occurs also have an influence.

1.6.2) Adhesion

Biofilm formation plays an important role in causing infections, and for biofilm formation, adhesion is necessary. It is known that a morphological change from the yeast cells to the filamentous stage (morphogenesis) increases adhesion of *Candida* species, therefore adhesion is seen as an important virulence factor (Cutler, 1991). For adhesion, several cell-surface molecules,

known as adhesins, were identified in this genus. These include mannoproteins and ligand-receptors on the outer surface of the organism, which may help *Candida* to bind to a substratum or cell surface (Vidotto *et al*., 2003). Ligand-receptors have been divided into different groups (Calderone & Braun, 1991). The first group includes molecules involved in the interactions between the protein fraction of a mannoprotein of *C. albicans* and the protein fraction of a host glycoprotein. The second group include molecules that are involved in the activity of lectins, where sugar moieties (fucosyl or N-acetyl-glucosamine) of the host-cell membrane glycoproteins are identified by the protein fraction of a *C. albicans* mannoprotein (Kanbe & Cutler, 1994). This type of adhesion is strain specific in *C. albicans*. Other factors that also play a role during adhesion include pH, temperature, phospholipase, protease and other extracellular enzymatic activities (Vidotto *et al*., 2003).

It is proposed that in *C. dubliniensis* the same mechanisms of adhesion apply, although it is not fully examined yet. However, it has been found that there are differences in the ability of these organisms to adhere to different substrates (Vidotto *et al*., 2003). This was illustrated by the adhesion of 27 *C. albicans* and 26 *C. dubliniensis* isolates from HIV positive patients to buccal and vaginal epithelial cells. From this study it was clear that both organisms adhered to these epithelial cells but that *C. albicans* was more adherent to both buccal and vaginal epithelial cells compared to *C. dubliniensis* cells. This could also be an indication of the higher virulence found in *C. albicans*. It was also found that *C. dubliniensis* was more adherent to vaginal than buccal epithelial cells. In contrast to this, Gilfillan *et al*. (1998) illustrated that oral *C. dubliniensis* isolates were more adherent to buccal epithelial cells when grown in glucose compared to *C. albicans*, but that the two species were equally adherent when grown in galactose. This might indicate that these two species are equally virulent, but at different sites in the host environment. Further studies need to be performed to clearly identify the differences in *C. albicans* and *C. dubliniensis* adhesion.

1.6.3) Hydrolytic enzymes

Lipolytic enzymes, such as lipase and phospholipase as well as secretory aspartyl proteinase may be produced to aid in the invasion of the host cells or to utilise host cell macromolecules as a source of nutrients (Fotedar & Al-Hedaithy, 2005; Ogawa *et al*., 1992). These enzymes damage and digest the cell membranes of the hosts which consist of proteins and lipids.

Lipases are water-soluble enzymes that belong to the esterase family of proteins and catalyse the hydrolysis of the ester bonds between fatty acids and glycerol (Bigey *et al*., 2003). Although lipase production and its role in fungal pathogens have received little attention, Fu *et al*. (1997) found that *C. albicans* produces and secretes lipases and Ogawa *et al*. (1992) indicated that pathogenic *C. albicans* isolates have a higher production of lipases compared to non-pathogenic isolates. This suggests that lipases might play a role in pathogenesis. Lipase production and its role in virulence in *C. dubliniensis* species have also not received much attention. However, Vidotto *et al*. (2004) indicated that both *C. albicans* and *C. dubliniensis* produced lipases but at low nano molar concentrations. Slifkin (2000) found that, after three days of incubation on Tween 80 medium, *C. albicans* had esterase activity and *C. dubliniensis* did not. Therefore, he suggested that the Tween 80 opacity test is very useful for differentiation between *C. albicans* and *C. dubliniensis*.

Phospholipase degrades the phospholipid constituents of the host cell membrane, which promotes invasion into host cells (Fotedar & Al-Hedaithy, 2005; Ogawa *et al*., 1992). Phospholipase in *C. albicans* was first reported in 1966, and it was the only *Candida* species considered to have this characteristic (Niewerth & Korting, 2001). Subsequently different phospholipases were identified in *C. albicans*, including phospholipase A, phospholipase B, phospholipase C, phospholipase D, lysophospholipase and lysophospholipase-transacylase. Subsequently, phospholipase production was also observed in *C. dubliniensis*, but at lower levels than *C. albicans* (Hannula *et al*., 2000). The difference in phospholipase production between these two species was used as a differentiation method. However, Vidotto *et al*. (2004) found that *C. dubliniensis* can also produce phospholipase at almost the same level as *C. albicans*. In contrast to this, Fotedar & Al-Hedaithy (2005) found that none of the 87 *C. dubliniensis* isolates tested produced phospholipase

and that all of the 52 *C. albicans* isolates tested produced phospholipase. The difference between the results obtained from these different researchers might be because of the different culture conditions used, variation within the different strains or more importantly the origin of the isolates.

The other important hydrolytic enzyme in virulence is proteinase. The function of proteinase in pathogenicity is considered to be enhancement of colonisation and penetration of the host tissue by the organism by hydrolysing the peptide bonds in the epithelial and mucosal barrier proteins such as collagen, keratin and mucin (Kantarcioğlu & Yücel, 2002). Another function of proteinase is to avoid the host's immune system by degrading a number of important proteins in the host defence mechanism such as immunoglobulins, complement system (number of small proteins found in the blood as part of larger immune system) and cytokines. Proteinase activity was observed in both *C. albicans* and *C. dubliniensis* (Vidotto *et al*., 2004). In this study all 27 tested *C. albicans* and 26 tested *C. dubliniensis* strains had this activity at similar levels. However, Gilfillan *et al*. (1998) found that *C. dubliniensis* has the ability to produce greater amounts of proteinase than some *C. albicans* strains. In contrast to these findings, Hannula *et al*. (2000) found that *C. albicans* isolates produced more proteinase than the *C. dubliniensis* isolates. This observed difference might be due to different numbers of isolates screened as well as the origin of the isolates. The strain or type of infection are not the only factors that influence proteinase production in *C. albicans*, but also phenotypic switch, environmental conditions and the phase of infection (De Bernardis *et al*., 2001).

1.6.4) Dimorphism

In yeasts, dimorphism or morphogenesis is the ability to adopt different morphologies and to grow either in the yeast or in the hyphal form (Chandra *et al*., 2001; Sullivan *et al*, 2004). This is also considered an important virulence factor of *C. albicans*, since biofilms consist out of complex combinations of cell types and it is known that the hyphal form of *C. albicans* is responsible for adherence and penetration of host tissues, although both forms have been observed to cause infection (Gilfillan *et al*., 1998; Ramage *et al*., 2005). Jabra-Rizk *et al*. (2004) stated that morphogenesis is triggered when *C. albicans*

comes into contact with a specific surface. There have been several factors identified that influence morphogenic conversion of *C. albicans*, ranging from chemicals to environmental conditions (Enjalbert & Whiteway, 2005). These factors include nutrient limitation, serum as nitrogen and carbon source, temperature, pH and low cell density.

Both *C. albicans* and *C. dubliniensis* have the ability to undergo morphogenic conversion and to produce different colony morphologies from smooth to rough colonies with hyphal fringes. However, it has been found that *C. dubliniensis* isolates switch faster than *C. albicans* isolates, thus having a high frequency of morphogenic conversion (Hannula *et al*., 2000).

Dimorphism in *C. albicans* and *C. dubliniensis* is regulated by quorum sensing (QS) (Enjalbert & Whiteway, 2005; Martins *et al*., 2007). Quorum sensing is the communication between cells that benefits the wellbeing of organisms in a biofilm by avoiding excessive overproduction and controlling competition for nutrients (Kruppa *et al*., 2004). This is made possible by the accumulation of small diffusible molecules, such as farnesol, that act as signals in the surrounding environment of the organism (Hogan, 2006). Farnesol is a 15-carbon isoprenoid with many important biological functions (Salonen & Vattulainen, 2005). One of these functions is as a QS signal of *C. albicans* and *C. dubliniensis*, specifically by *E*,*E*-farnesol (Henriques *et al*., 2007; Hornby *et al*., 2001). It inhibits morphogenesis (yeast-to-hyphae transition) and biofilm formation during high-density growth, allowing the culture to grow as actively budding yeasts (Kruppa *et al*., 2004; Ramage *et al*., 2002). Even at low cell densities, exposure to farnesol will inhibit germ tube formation. Interestingly, Martins *et al*. (2007) identified a group of extracellular alcohols, produced by both planktonic cells and biofilms of *C. albicans* and *C. dubliniensis*, which also acts as signalling molecules. These molecules were identified as isoamyl alcohol, 2-phenylethanol, 1-dodecanol and *E*-nerolidol. It was found that these molecules inhibit morphogenesis of both *C. albicans* and *C. dubliniensis* at different concentrations.

Another active compound that is continuously released during growth of *C. albicans* was identified as 2-(4-hydroxyphenyl) ethanol or tyrosol (Chen *et al*., 2004). This compound is a derivative of the amino acid tyrosine and its function is to accelerate germ tube and hyphal formation. Presently, nothing is

known about the production of tyrosol by *C. dubliniensis*. The activities of farnesol, the group of extracellular alcohols and tyrosol indicate that morphogenesis is under positive and negative control.

Cell surface hydrophobicity, adhesion, secretion of hydrolytic enzymes and dimorphism play important roles in biofilm formation (Ramage *et al*., 2001a). The biofilm environment not only gives the organism the necessary nutrients needed for growth, but also gives protection against the host immune response and certain drugs used in antifungal therapy. Biofilm formation might therefore be one of the reasons why antifungal drug resistance is a characteristic of both *C. albicans* and *C. dubliniensis*.

1.7) Antifungal drug resistance

Resistance is defined as the competency of a microorganism to grow in the presence of a high level of an antimicrobial agent (Lewis, 2001), and clinical resistance is the perseverance of clinical lesions even though the prescribed dose, known to be effective, is used for at least seven days, and this is often associated with biofilm formation (Al-Mohsen & Hughes, 1998). Since both *C. albicans* and *C. dubliniensis* have the ability to form biofilms, these infections may exhibit increased resistance to antifungal treatment and host immune defences (Jabra-Rizk *et al*., 2004; Ramage *et al*., 2001b). Antifungal resistance of biofilms may be attributed to a restriction in penetration of the drug, due to the negatively charged EXM (Lewis, 2001). Another factor is a decrease in the growth rate of the cells in a biofilm, because most antimicrobials are active on fast growing organisms. The expression of resistance genes, such as those that encode the proteins for the multidrug resistance pumps which transport the antimicrobials across the cell membrane might also be involved in resistance. Presently, there are two main groups of drugs used for the treatment of antifungal infections, namely drugs produced by organisms, e.g. the polyene drugs, amphotericin B and nystatin, and the synthetically produced drugs, e.g. flucytosine and the azole drugs (Al-Mohsen & Hughes, 1998). The polyene antifungal, amphotericin B, which is fungicidal and has the broadest spectrum of antifungal activity, has become the “gold standard” for the treatment of mycoses (Perfect & Wright, 1994; Mahmoudabadi & Drucker, 2006).

Amphotericin B binds hydrophobically to ergosterol, a major component of fungal cell membranes, changing the membrane permeability and integrity by forming pores, in which the polyene hydroxyl residues face inward, leading to vital cytoplasmic leakage and fungal cell death (Al-Mohsen & Hughes, 1998; Graybill, 2000) (Fig. 15). However, problems arise with the toxicity, especially nephrotoxicity, the development of resistance and the non-availability of an effective oral form for long-term therapy of amphotericin B. To overcome these problems, amphotericin B has been formulated into liposomes or attached to lipid vehicles to allow the transfer of higher doses of amphotericin B and reducing the toxicity to mammalian cells (Al-Mohsen & Hughes, 1998; Ghannoum & Rice, 1999).

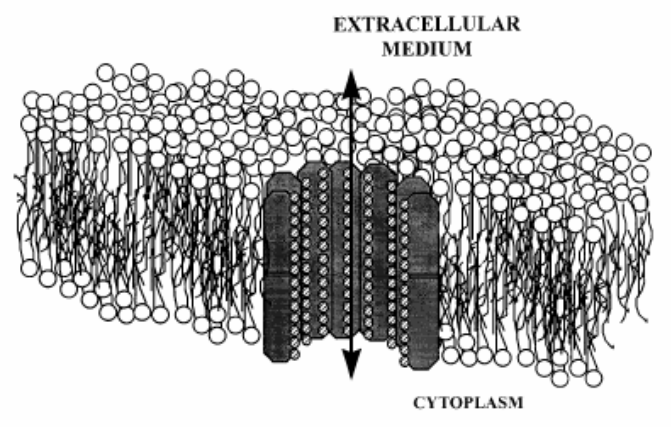


Fig. 15. The interaction between amphotericin B and cholesterol in a phospholipid bilayer forms a conducting pore through which the cytoplasmic contents leak out (Ghannoum & Rice, 1999).

Other lipophilic antifungals often used to treat superficial infections, are the azoles and include the imidazoles (clotrimazole, miconazole and ketoconazole) and the triazoles (fluconazole and itraconazole) (Jabra-Rizk *et al*., 2004; Mahmoudabadi & Drucker, 2006). These antifungals increase the membrane permeability and instability by inhibiting the cytochrome P450-dependent enzyme, 14-α-demethylase, responsible for the demethylation of lanosterol to ergosterol (Al-Mohsen & Hughes, 1998; Como & Dismukes, 1994; Graybill, 2000) (Fig. 16). This inhibition leads to the depletion of ergosterol and the accumulation of sterol precursors, changing the structure and function of the plasma membrane. The azole drugs have a lower toxicity

compared to the polyenes, and their action is slow compared to the polyenes. The azole drugs are primarily used for the treatment of mucosal candidiasis in HIV positive patients, however, resistance to these drugs developed due to the increased use of these fungistatic drugs (Jabra-Rizk *et al*., 2004).

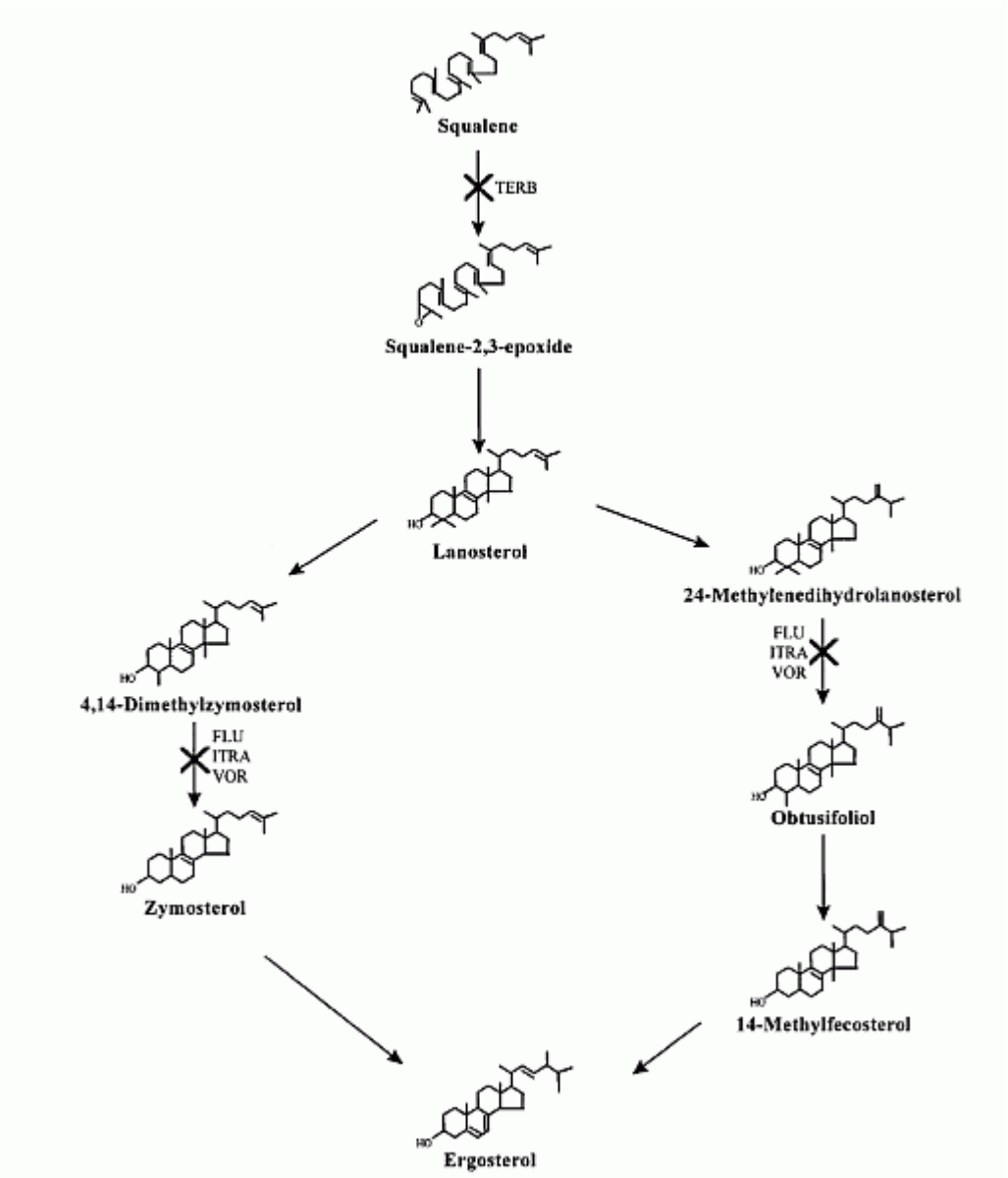


Fig. 16. Ergosterol biosynthetic pathway indicating different steps where antifungal agents can inhibit the pathway. FLU, fluconazole; ITRA, itraconazole; TERB, terbinafine; VOR, voriconazole (Ghannoum & Rice, 1999).

It is known that *C. albicans* is resistant to these clinically important antifungals including, fluconazole, flucytosine, itraconazole and ketoconazole especially in the biofilm state (Al-Fattani & Douglas, 2004). *Candida krusei* and *C. glabrata*, similar to *C. albicans*, show an increased resistance to fluconazole and clinical isolates of *C. dubliniensis* develop resistance to fluconazole *in vitro* when exposed to the drug (Bennett *et al*., 2004; Moran *et al*., 1997; Singh *et al*., 2002). Interestingly it was found that after treatment of *C. dubliniensis* with fluconazole there was an increase in adherence to epithelial cells and an increase in proteinase production (Borg-Von Zepelin *et al*., 2002). The opposite was observed for *C. albicans*, which might give *C. dubliniensis* a growth advantage over *C. albicans* in patients treated with fluconazole.

It is hypothesised that resistance to polyene antifungals is due to changes in the sterol content of the cells, in other words resistant cells with a changed sterol content should bind smaller amounts of polyene than do susceptible cells (Hamilton-Miller, 1973). This was indicated by Dick *et al*. (1980), when they studied polyene resistant *C. albicans* strains from clinical samples, and found that 74 to 85 % of the 27 resistant isolates had decreased ergosterol content. This was also found true for *C. lusitaniae* with a decrease in ergosterol content as well as changes in the sterol composition for strains resistant to amphotericin B (Peyron *et al*., 2002).

The development of resistance to azole drugs might be due to several mechanisms (Fig. 17), such as modification of the target enzyme, for example by mutations (Ghannoum & Rice, 1999). Other possible mechanisms include an active efflux system, such as the major facilitator superfamily (MFs) and the adenosine triphosphate-binding cassette (ABC) (Pinjon *et al*., 2005). *Candida albicans* and *C. dubliniensis* have both these two types of drug efflux pumps (White *et al*., 1998). The ABC transporters are involved in removing azole drugs and MFs are responsible for removing tetracycline type drugs out of the cell. Other mechanisms involved in developing drug resistance include a decreased access to the target enzyme, made possible by a reduction in the intracellular concentration of the enzyme, and changes in the ergosterol biosynthetic pathway (Pinjon *et al*., 2005).

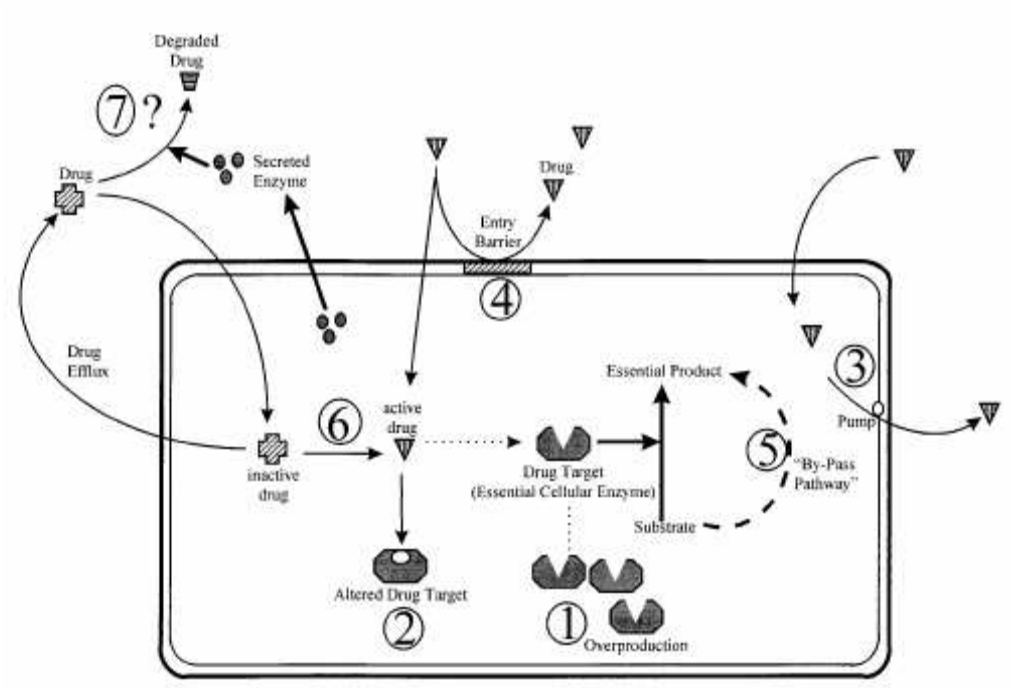


Fig. 17. Mechanisms of drug resistance by microbial cells. 1) Overproduction of target enzyme. 2) Alteration of target drug so that it cannot bind to the target. 3) Efflux pump removes drug. 4) The drug cannot enter the cell at the cell membrane/cell wall level. 5) Bypass pathway of cell that compensates for the loss-of-function inhibition due to the drug activity. 6) Inhibition of some fungal enzymes that convert an inactive drug to its active form. 7) Degradation of the drug by enzymes secreted by the cell to the extracellular medium (Ghannoum & Rice, 1999).

1.8) Lipids in yeasts

Lipids are one of the natural products, together with proteins and carbohydrates, which have been studied for centuries (Stodola *et al*., 1967). Lipids are classified as a diverse group of fatty substances found in all living organisms and believed to play a role as long-term storage material for energy in the form of triacylglycerols as well as structural role in biological membranes and influence the interaction with certain antimicrobial drugs (Ballmann & Chaffin, 1979; Kitamoto *et al*., 1992). Today lipids are also known to be involved in more complex functions, such as the control of biological processes by acting as signalling molecules (Shea & Del Poeta, 2006). Lipids are also indirectly involved as one of the many virulence factors in disease causing organisms by regulating the development of the infectious process

(Mahmoudabadi *et al*., 2002; Mishra *et al*., 1992; Noverr *et al*., 2003). As mentioned earlier, adhesion is an important step in biofilm formation and the latter is known to be important in pathogenesis. Ghannoum *et al*. (1986) stated that lipid classes such as phospholipids, sterols and steryl esters play an important role during adherence of *Candida* species.

It is known that morphogenesis is an important characteristic of *Candida* species and is believed to be one of the factors influencing pathogenicity (Gilfillan *et al*., 1998; Ramage *et al*., 2005). In some *Candida* species temperature, pH and carbon source influence the production of yeast cells and mycelium, but what is more important are that these parameters also affect the lipid composition (Mahmoudabadi *et al*., 2002). It was found that there is a difference in the lipid content between the yeast and mycelium forms, with an increasing amount of lipids from yeast to mycelial form. In addition, it has been shown that there is a relationship between morphogenesis and sterol composition in these pathogenic organisms, especially in the amount of ergosterol, the major component in the membrane (Cannon & Kerridge, 1988; Shimokawa *et al*., 1986).

Another lipid component that may influence pathogenesis is phospholipids (PLs), because of its importance in membrane function and cell morphology, as well as the interaction with antifungal drugs (Mishra *et al*., 1992). Most antifungals target the cell membranes (e.g. polyene drugs) or have to cross it to reach its target site (e.g. azole drugs). It was found that an increase in saturation of lipids causes resistance to antifungal drugs, specifically to aculeacin, which inhibits glucan synthesis. Therefore, to study the lipids in pathogenic fungi might help in developing novel antifungal agents.

1.8.1) Eicosanoid production

Eicosanoids represent a large group of oxygenated C20 fatty acids and are made up of three groups: the prostanoids (prostaglandins and thromboxanes), the leukotrienes and related hydroxy fatty acids, and the epoxides (Smith, 1989). All three groups are synthesised by different pathways, the cyclo-oxygenase-, lipoxygenase- and an epoxygenase pathway respectively. These eicosanoids can be synthesised from polyunsaturated fatty acids found naturally in the environment. Arachidonic acid (*all-cis*-5,8,11,14-

eicosatetraenoic acid) (20:4) is a polyunsaturated omega-6 fatty acid ($C_{20}H_{32}O_2$), present in the PLs of human cells (Noverr *et al*., 2003). It is an important precursor in the production of bioactive eicosanoids, such as prostaglandins and leukotrienes by the mammalian cyclooxygenase (COX-1 and COX-2) and lipoxygenase enzymes (Fig.18).

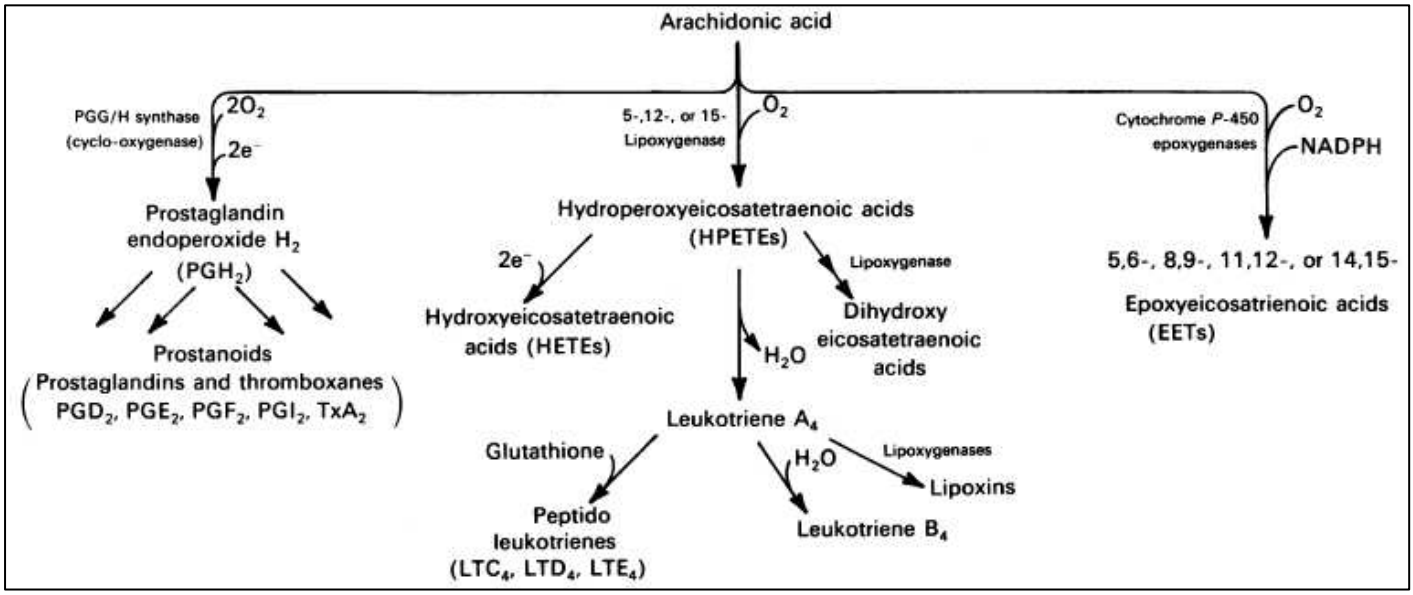


Fig. 18. The arachidonate cascade illustrating the production of eicosanoids from the oxygenation of arachidonic acid (Smith, 1989).

During *C. albicans* infection, 20:4 is released from the PLs of the membrane of infected host cells by fungal phosholipases (Alem & Douglas, 2004; Deva *et al*., 2001) (Fig. 19). *Candida albicans* has the ability to utilise 20:4 as sole carbon source to stimulate cell growth and morphogenesis (Deva *et al*., 2000). Ciccoli *et al*. (2005) hypothesised that *C. albicans* converts 20:4 to a 3(R)-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid, which then serves as a substrate for the production of the inflammatory 3-hydroxy- prostaglandin E_2 (3-OH PGE_2) by the host cells. 3-hydroxy PGE_2 has immunosuppressive activities and enhances vascular permeability in the host, which may weaken the host defences and facilitate the invasion of the host tissue/cells (Deva *et al*., 2001). *Candida albicans* can also use exogenous 20:4 as a precursor for the production of bioactive eicosanoids, such as prostaglandin E_2 (PGE_2). It was shown that PGE_2 can inhibit TH1-type immune responses responsible for the evolution of cell-mediated immune responses by inflammation (Chen *et al*.,

2000b), chemokine production, phagocytosis and lymphocyte proliferation (Noverr *et al*., 2002) as well as promoting TH2-type responses, tissue eosinophilia and immunoglobulin E production (Erb-Downward & Noverr, 2007). Both endogenous eicosanoids and 3-OH PGE_2 are known as important virulence factors (Alem & Douglas, 2004; Erb-Downward & Huffnagle, 2006; Erb-Downward & Noverr, 2007; Noverr *et al*., 2002). In addition to prostaglandins, Deva *et al*. (2000) discovered a novel eicosanoid, 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid, in *C. albicans*, produced from exogenous 20:4. However, the role of this eicosanoid in pathogenicity is still unknown.

In contrast to all the information available about eicosanoid production by *C. albicans*, there is no literature regarding eicosanoid production by *C. dubliniensis*.

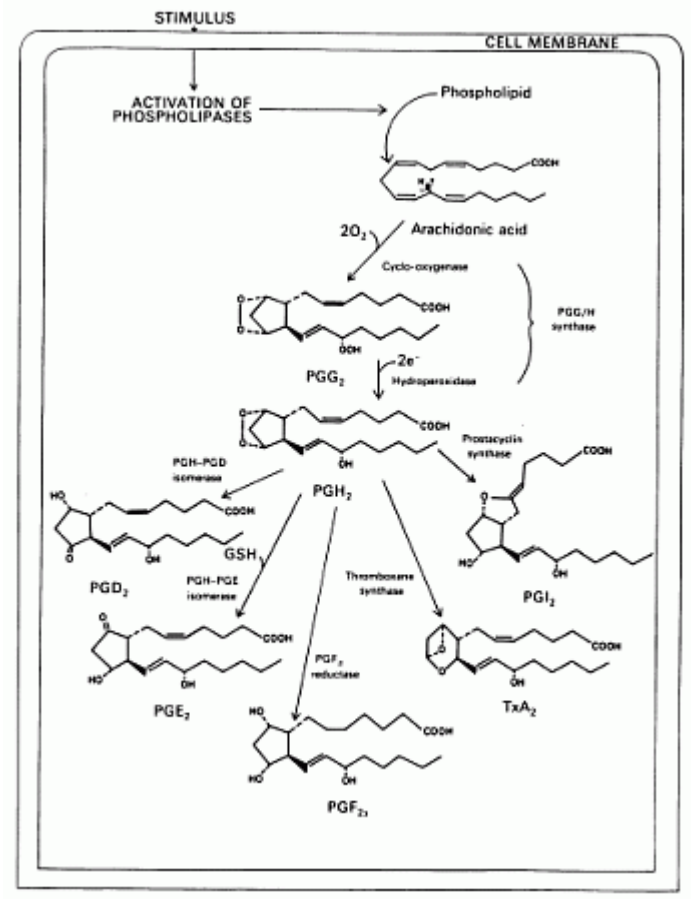


Fig. 19. Biosynthetic pathway illustrating the production of prostanoids from arachidonic acid liberated from the phospholipids in cell membrane by phospholipase activity (Smith, 1989).

1.9) Purpose of study

With this as background the aim of this study became:

1) To use known phenotypic identification techniques to characterise *C. albicans* and *C. dubliniensis* strains in order to select phenotypically different strains for further studies (Chapter 2).

2) To evaluate the effect of exogenous arachidonic acid on growth and biofilm formation of the selected *C. albicans* and *C. dubliniensis* strains (Chapter 3).

3) To evaluate the uptake and incorporation of arachidonic acid in the selected *C. albicans* and *C. dubliniensis* strains and to evaluate the ability of selected strains to produce eicosanoids in the presence of arachidonic acid (Chapter 4).

4) To evaluate the susceptibility of selected *C. albicans* and *C. dubliniensis* strains to antifungals (amphotericin B and clotrimazole) after treatment with arachidonic acid (Chapter 5). This combination may result in the reduction of the amount of antifungal needed to inhibit *Candida* biofilms, leading to lowered toxicities and economical advantages.

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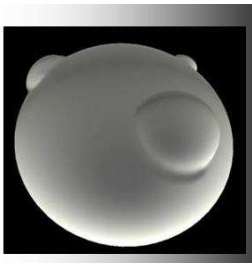
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CHAPTER 2

Characterisation of *Candida albicans* and *C. dubliniensis* strains

2.1) Abstract

Candida albicans and *C. dubliniensis* are two closely related species that are important opportunistic pathogens causing serious infections. These serious infections are increasing because of the increase in immunocompromised patients. *Candida albicans* and *C. dubliniensis* share many phenotypic characteristics that make it difficult to differentiate between them, especially in a clinical sample. As a result several identification techniques, based on phenotypic characteristics, have been developed to differentiate between these *Candida* species. The aim of this study was to use current identification techniques to characterise selected *C. albicans* and *C. dubliniensis* strains in order to select strains that are phenotypically dissimilar. The methods used in this study include fluorescence on methylene blue agar plates, chlamydospore production on tobacco and sunflower seed husk agar plates, the germ tube test and growth at elevated temperatures. The growth of planktonic cells as well as biofilm production was also determined by the Klett-Summerson photoelectric colorimeter and by the use of crystal violet stain respectively. Virulence factors that have been studied include the production of hydrolytic enzymes i.e. lipase and phospholipase activity as well as the percentage hydrophobicity. The results obtained indicated that there is overlap in phenotypic characteristics of these species. However, it was possible to select three phenotypically dissimilar strains.

2.2) Introduction

Candida infections are increasing due to the increasing incidence of HIV infection, the use of antimicrobial drugs, cancer chemotherapy, the use of immunosuppressive agents and the increase in the use of implanted medical devices (e.g. catheters) (Xiaogang *et al*., 2003). *Candida* species were the fifth most common organism isolated from blood and the seventh most common cause of nosocomial infections in the United States in the eighties, but due to the increasing frequency in HIV infected individuals in the nineties it increased to the fourth most commonly isolated pathogen in immunocompromised persons (Girishkumar *et al*., 1999; Pfaller & Diekema, 2007).

Candida albicans is the most important and most frequently isolated pathogen responsible for these infections (Girishkumar *et al*., 1999; Pfaller & Diekema, 2007). However, during the last decade other *Candida* species also became clinically important with approximately 50 % of reported cases of fungemia among HIV infected children being caused by non-*albicans Candida* species (Sullivan *et al*., 2004). It has been shown that most people carry only one strain of *Candida* at different body sites. However, immunocompromised and hospitalised individuals may carry more than one strain or species of *Candida* (McCullough *et al*., 1996; Soll *et al*., 1988).

Candida dubliniensis share many phenotypic characteristics with *C. albicans* and as a result was previously misidentified as *C. albicans* (Sullivan *et al*., 1995). However, there are some differences between these two species that enable differentiation of these species using different methods, both genetic and phenotypic. The different methods based on phenotypic characteristics include morphology on Pal's agar (sunflower seed agar) (Mosaid *et al*., 2003), growth at 42-45 ℃ (Pinjon *et al*., 1998), fluorescence on methylene blue-Sabouraud agar (Yücesoy *et al*., 2001) and their carbon assimilation profiles (Sullivan *et al*., 1995). All these methods have their advantages and disadvantages and should usually be used together to avoid false results.

The aim of the study was to use standard identification techniques to differentiate between selected strains of *C. albicans* and *C. dubliniensis*. This would allow the selection of phenotypically dissimilar strains for further studies.

2.3) Materials and methods

2.3.1) Strains used

The strains used in this study and their origins are listed in Table 1. All strains were maintained on yeast malt extract (YM) agar [10 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone, 16 g agar, 1000 mL distilled water (dH_20)] at room temperature.

Table 1: The origin of the *Candida* strains used.

Strains	Source
C. albicans CBS 562T	Interdigital mycosis (CLN), Uruguay
C. albicans NRRL Y-0477	Human faeces (CLN), unknown
C. albicans NRRL Y-27088	Rectum of pregnant woman (CLN), Denmark
C. albicans NRRL Y-27077	Skin lesions (CLN), Germany
C. dubliniensis NRRL Y-17841T	Oral cavity of HIV infected patient (CLN), Ireland
C. dubliniensis NRRL Y-27787	CLN, USA
C. dubliniensis NRRL Y-27853	CLN, USA

CLN, clinical strain

2.3.2) Methylene blue agar plates

Candida strains were grown for 24 h on YM agar plates at 30 ℃. This assay was performed according to Goldschmidt *et al*. (1991) but with slight modifications. The strains were aseptically transferred to YM agar plates containing 0.01 % (w/v) methylene blue dye. The plates were incubated at 30 ℃ for 24 h and then visualised under long wave len gth UV light (365 nm) for fluorescence. This experiment was performed in duplicate.

2.3.3) Tobacco agar plates

Candida isolates from 24 h old cultures on YM agar plates, were aseptically streaked out on tobacco agar plates. The plates were prepared by adding 50 g cigarette tobacco (Marlboro) to 1000 mL of dH_20 and boiling for 30 min. The infusion was filtered through filter paper and the volume adjusted to 1000 mL with dH_20. Twenty gram agar was added and the pH adjusted to 5.4 with 1 mM HCl. The media was then autoclaved at 121 ℃ for 15 min (Khan *et al*., 2004; Tendolkar *et al*., 2003). After aseptically streaking out a small amount of the different inocula from the YM plates, the plates were incubated at 30 ℃ and observed daily for four days for different colony characteristics. These included surface topography (rough or smooth), colony colour, the formation of hyphal fringes and the formation of chlamydospores. The latter was performed using a Zeiss Axioplan light microscope coupled to a Colorview soft imaging system directly at low magnification (100X) for hyphal fringe production and at

high magnification (400X, 1000X) for chlamydospore production. This experiment was performed in duplicate.

2.3.4) Sunflower seed husk agar plates

Candida cultures, grown for 24 h on YM agar plates, were aseptically streaked onto sunflower seed (*Helianthus annuus*) husk (SSH) agar plates. This medium contained 50 g pulverised sunflower seed husk (manually removed from the seed) which was boiled for 30 min in dH_20. The infusion was filtered through filter paper and 10 g glucose and 16 g agar were added to the extract. The volume was adjusted to 1000 mL with dH_2O and the pH to 5.5 with 1 mM HCl, followed by autoclaving at 121 ℃ for 15 min (Khan *et al*., 2005). After the plates were inoculated, a sterile microscope slide was placed over the inoculated yeast, creating anoxic conditions according to the Dalmau plate technique (Yarrow, 1998). The plates were then incubated at 30 ℃ and the colonies examined daily for three days for surface topography (rough or smooth), colony colour, the formation of hyphal fringes and the formation of chlamydospores. The latter was performed using a Zeiss Axioplan light microscope coupled to a Colorview soft imaging system directly at low magnification (100X) for hyphal fringe production and at high magnification (400X, 1000X) for chlamydospore production. This experiment was performed in duplicate.

2.3.5) Germ tube test

A small portion of a pure colony of 18-24 h old *Candida* culture on YM agar plates was used to inoculate sterile test tubes containing 0.5 mL foetal bovine serum albumin (Sigma-Aldrich, USA). The tubes were incubated aerobically at 37 ℃ on a shaker. The cultures were microscopicall y investigated with a Zeiss Axioplan light microscope coupled to a Colorview soft imaging system at 30 min, 45 min, 60 min, 90 min and 120 min intervals. Appearance of small filaments projecting from the cell surface confirms germ tube formation (Elmer *et al*., 1992). This experiment was performed in duplicate.

2.3.6) Growth at an elevated temperature

Candida cultures, grown for 24 h on YM agar plates, were aseptically streaked onto fresh YM agar plates and incubated for 48 h at 45 ℃ (Pinjon *et al*., 1998). Growth was visually determined. This experiment was performed in duplicate.

2.3.7) Lipase activity assay

The different *Candida* strains were grown in 10 ml sterile yeast nitrogen base (YNB) broth [6.7 g YNB, 1000 mL dH_2O] containing 10 $g.L^{-1}$ glucose in sterile centrifuge tubes for 24 h at 37 ℃. The cells were washed with phosphate buffered saline (PBS) (OXOID, UK), counted with a hemacytometer and diluted to 1 x 10^6 $cells.mL^{-1}$ in sterile PBS. Tributyrate agar was prepared by sonicating 5 ml tributyrate, 5 g gum arabic and 50 mL dH_20 for 2 min to a milky emulsion with a sonifier cell disruptor (Bandelin Sonoplus UW 2070, Berlin, Germany) and adding the emulsion to 2.5 g peptone, 1.5 g yeast extract, 6 g agar and 450 mL dH_20. The pH was adjusted to 8.0 with 1 mM NaOH before autoclaving (Kouker & Jaeger, 1987). To quantify lipase activity, 9 mm diameter wells were punched into the tributyrate agar and 100 µL of the 1 x 10^6 $cells.mL^{-1}$ suspension was added to each well. The plates were left to dry at room temperature and then incubated at 37 ℃. The clearance zones were measured after 24 h until the zones reached a maximum diameter. The diameters of the colonies and the colony-plus-clearance zones were measured. The diameter of the colony was then divided by the diameter of the colony-plus-clearance zone (Price *et al*., 1982). This value is known as the Pz value. This experiment was performed in triplicate.

2.3.8) Phospholipase activity assay

For the preparation of the phospholipase medium (egg yolk medium), 2 % (w/v) Sabouraud's dextrose agar (20 g glucose, 10 g peptone, 16 g agar, 1000 mL dH_2O), 11.7 g NaCl and 0.11 g $CaCl_2$ were added to 184 mL dH_2O and then autoclaved. To this sterile cooled medium a 10 % sterile egg yolk emulsion (MERCK, SA), centrifuged for 10 min at 500 *g*, was added (Kadir *et al*., 2007). The different *Candida* strains were grown and washed the same as for the lipase activity with the only difference being that the cell concentrations

used was 1 x 10^7 cells.mL^{-1}. The plates were left to dry at room temperature and then incubated at 37 ℃. The zones of precipita tion were measured from 24 h until the zones reached a maximum diameter (Kadir *et al*., 2007). The zone diameters were also interpreted according to Price *et al*. (1982). This experiment was performed in triplicate.

2.3.9) Hydrophobic microsphere assay

The different *Candida* strains were grown in 2 % (w/v) Sabouraud's dextrose broth (20 g glucose, 10 g peptone, 1000 mL dH_2O) at 25 ℃ and 37 ℃ for 24 h. The cells were then washed with cold deionised water, counted with a hemacytometer and diluted to 5 x 10^6 cells.mL^{-1} in ice cold sodium phosphate buffer (PUM buffer) (Hazen & Hazen, 1987). The PUM buffer was prepared by adding 22.2 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea and 0.2 g $MgSO_4 \cdot 7H_2O$ to a final volume of 1000 mL dH_2O and the pH adjusted to 7.1 with 1 mM NaOH. The PUM buffer was homogenised for 2 min with a sonifier cell disruptor (Bandelin Sonoplus UW 2070, Berlin, Germany) (Powell *et al*., 2003). The cell suspensions were kept at 0 ℃ until used (within 1 h) (Hazen & Hazen, 1987). From a stock solution of polystyrene microspheres (diameter 0.80 µm) (Bangs Laboratories, Inc.) with a low negative charge density, 6 µL were mixed with 2 mL of PUM buffer. These suspensions were kept in glass test tubes, which were washed with concentrated HCl, and kept at 0 ℃ until used (within 1 h). One hundred and fifty microlitres of each cell suspension was mixed with 150 µL of the microsphere suspension in glass test tubes which were washed with concentrated HCl. The test tubes were left at room temperature for 2 min and then vortexed for 30 sec. The percentage hydrophobicity of each organism was determined microscopically by counting at least 200 colony forming units (cfu) having three or more microspheres attached and dividing it by the total number of cfu. This was performed in duplicate with a Zeiss Axioplan light microscope coupled to a Colorview soft imaging system.

2.3.10) Growth of planktonic cells

The *Candida* strains were grown on YM agar plates for 24 h at room temperature. The strains were then inoculated into 200 ml sterile YNB broth containing 10 g.L^{-1} glucose (pre-inoculum) in 1000 mL conical flasks and

incubated aerobically at 37 ℃ for 12 h. The cells were diluted to 10 Klett units on a Klett-Summerson photoelectric colorimeter in 100 mL sterile YNB broth containing 10 $g.L^{-1}$ glucose in 500 mL conical flasks and incubated aerobically at 37 ℃. The optical density (OD) of the suspension was measured in Klett units every 2 h for up to 20 h. From the obtained data, growth curves were constructed. This was performed in triplicate.

2.3.11) Biofilm production

The *Candida* strains were grown on YM agar plates for 24 h at room temperature. The strains were inoculated into 10 mL sterile YNB glucose broth containing 10 $g.L^{-1}$ glucose in 50 mL sterile centrifuge tubes and incubated at 30 ℃ for 48 h. The cells were harvested by centrifugation for 5 min at 4000 *g* with a Heraeus® Megafuge® 1.0R (UK) centrifuge and washed twice with sterile PBS and resuspend in 5 ml filter sterilised RPMI-1640 medium (Sigma-Aldrich, USA). The cells were counted with a hemacytometer and diluted to 1 x 10^6 $cells.mL^{-1}$ in 5 mL filter sterilised RPMI-1640 medium. From the diluted cell suspension, 100 µL was dispensed into each well of a 96-well microtiter plate (Corning Incorporated, Costar®, USA). The negative control wells contained only 100 µL RPMI-1640 medium. The 96-well microtiter plates were incubated at 37 ℃ for 1 h, 8 h, 24 h, 48 h and 72 h respectively in order to allow the quantification of biofilm formation over time in terms of biomass (Jin *et al*., 2003). After the incubation period the wells were carefully washed twice with 200 µL sterile PBS to remove planktonic cells (free-floating cells) and the medium. The plates were air dried for 45 min and then each well was stained with 110 µL of 1 % (v/v) aqueous crystal violet solution for 45 min. The wells were again washed with sterile dH_2O until no purple colour could be extracted from the wells. Each well was immediately destained with 200 µL of 95 % ethanol for 45 min and 100 µL of the destaining solution was then transferred to a new well. The amount of crystal violet stain in the destaining solution was then measured as OD at 600 nm with a Labsystems iEMS Reader (Thermo Bioanalysis, Helsinki, Finland). The absorbance values of the control wells were subtracted from the values of the test wells. This was performed in triplicate.

2.3.12) D1/D2 Sequencing

DNA was extracted from *Candida albicans* NRRL Y-0477 and molecular identification was performed through sequence analysis of the D1/D2 domain of the 26S ribosomal DNA. PCR was performed on the extracted DNA using primer pair NL-1 (5'-gcatatcaataagcggaggaaaag) and NL-4 (5'-ggtccgtgtttcaagacgg) (Kurtzman & Robnett, 1998). A total of 30 PCR cycles were performed with initial denaturation at 94 ℃ for 2 min, denaturation at 94 ℃ for 30 sec, annealing at 50 ℃ for 30 sec and extension at 72 ℃ for 1 min. A final extension was carried out at 72 ℃ for 5 min. A 1.0 % (w/v) agarose gel, with ethidium bromide incorporated, was used to confirm the PCR product which was visualised under UV light. A commercial ladder was used to determine the sizes of the PCR products.

After purification, sequencing reactions were performed with the ABI Prism™ Big Dye terminator™ V3.1 cycle sequencing ready reaction kit and data collected on an ABI 3130XL genetic analyzer (Applied biosystems). Data were analysed using data collection software V3.0 and sequences assembled using VectorNTI ver 10. The obtained DNA sequence was compared to a database of sequences by the use of the BLAST program.

2.4) Results and discussion

2.4.1) Methylene blue agar plates

The methylene blue agar plate method is very useful to differentiate between *C. albicans* and *C. dubliniensis* strains, especially in clinical settings (Schoofs *et al*., 1997). *Candida albicans* strains were found to fluoresce whereas *C. dubliniensis* strains do not fluoresce under Wood's light (long wave length UV) (Goldschmidt *et al*., 1991; Schoofs *et al*., 1997). The reason for the fluorescence is still unknown, but it is postulated that there might be a reaction with specific cell wall polysaccharides (Yücesoy *et al*., 2001). In this experiment it was found that all the *C. albicans* strains fluoresced under long wave UV light, except for *C. albicans* NRRL Y-0477, and all the *C. dubliniensis* strains tested did not fluoresce under the same conditions (Fig. 1). The illustrated variation between *C. albicans* strains might be explained by the fact that *C. albicans* strains might lose their fluorescence and *C. dubliniensis* might

gain fluorescence on this medium after subculturing and storage, which may make differentiation even more difficult (Schoofs *et al*., 1997; Sullivan & Coleman, 1998). Thus, this medium may only be useful for differentiation of primary cultures i.e. after initial isolation of a *Candida* culture.

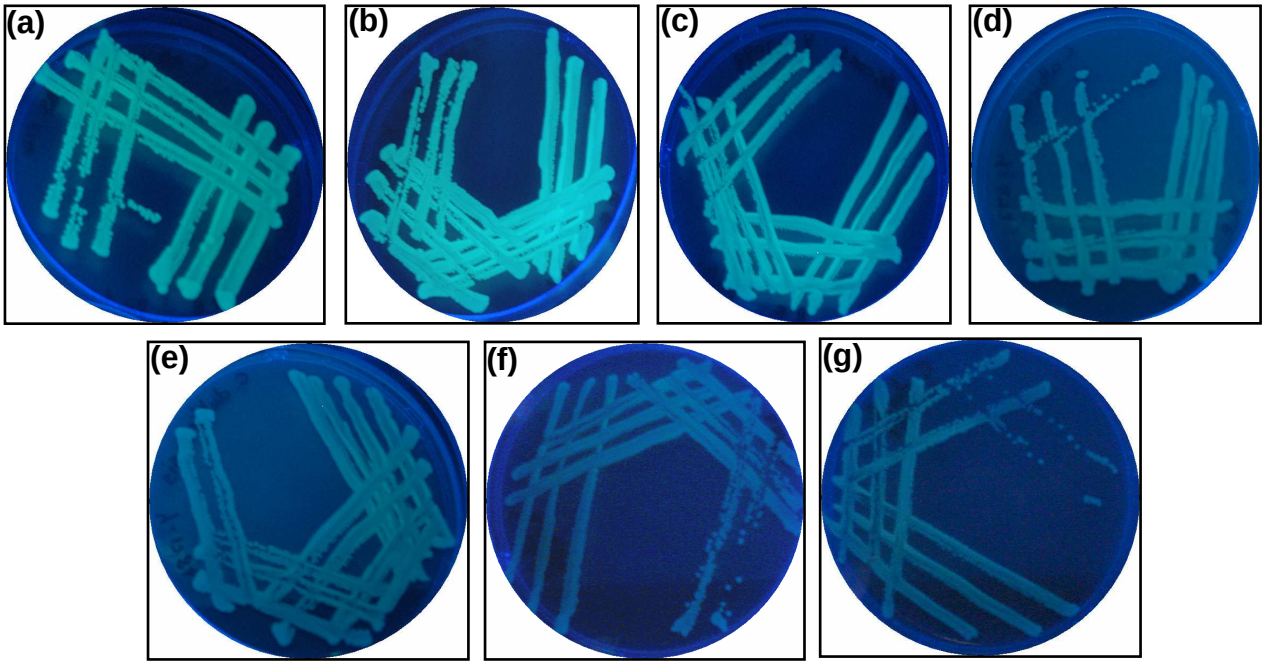


Fig. 1. Methylene blue agar plates indicating the fluorescence or non-fluorescence of *Candida* strains after 24 h of incubation at 30 ℃. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-27077. (c) *C. albicans* NRRL Y-27088. (d) *C. albicans* NRRL Y-0477. (e) *C. dubliniensis* NRRL Y-17841T. (f) *C. dubliniensis* NRRL Y-27787. (g) *C. dubliniensis* NRRL Y-27853.

2.4.2) Tobacco agar plates

Tobacco agar is used for the differentiation of *C. albicans* from *C. dubliniensis* strains by the formation of chlamydospores and hyphal fringes by the latter but not by *C. albicans* strains (Khan *et al*., 2004). Advantages of this medium is that it is inexpensive to produce and useful for rapid identification and characterisation of a large number of samples.

From the results obtained it was found that three of the four *C. albicans* strains tested (i.e. *C. albicans* CBS 562T, *C. albicans* NRRL Y-27077 and *C. albicans* NRRL Y-27088) produced smooth white colonies, without hyphal fringes or chlamydospores (Fig. 2a, b, c). *Candida albicans* NRRL Y-0477 (Fig. 2d) had

the same characteristics as the *C. dubliniensis* strains tested (i.e. *C. dubliniensis* NRRL Y-17841T, *C. dubliniensis* NRRL Y-27787 and *C. dubliniensis* NRRL-27853) with the production of cream to brown colonies and hyphal fringes which contained chlamydospores after 72 h of incubation at 30 ℃ (Fig. 2e, f, g). These results agree with the wo rk of Kumar & Menon (2005) who found that both *C. albicans* and *C. dubliniensis* have the ability to produce hyphal fringes and chlamydospores on this medium. From their results they concluded that tobacco agar is good for the differentiation of *C. albicans* and *C. dubliniensis* species from other *Candida* species. Sullivan *et al*. (1995) indicated that *C. dubliniensis* forms pairs or triplets of chlamydospores on the ends of short-branched hyphae and that *C. albicans* forms a single chlamydospore on the tip of a suspensor cell. However in this study, it was found that the *C. dubliniensis* strains tested also produced single chlamydospores on the tips of the cell (Fig. 2e, f, g). This agrees with what Ellepola *et al*. (2003) found on corn meal-Tween agar, that *C. dubliniensis* showed no constant pattern of chlamydospore formation. Care should be taken when using chlamydospore production for differentiation because of variation between strains, as indicated by *C. albicans* NRRL Y-0477 which resembles *C. dubliniensis* strains.

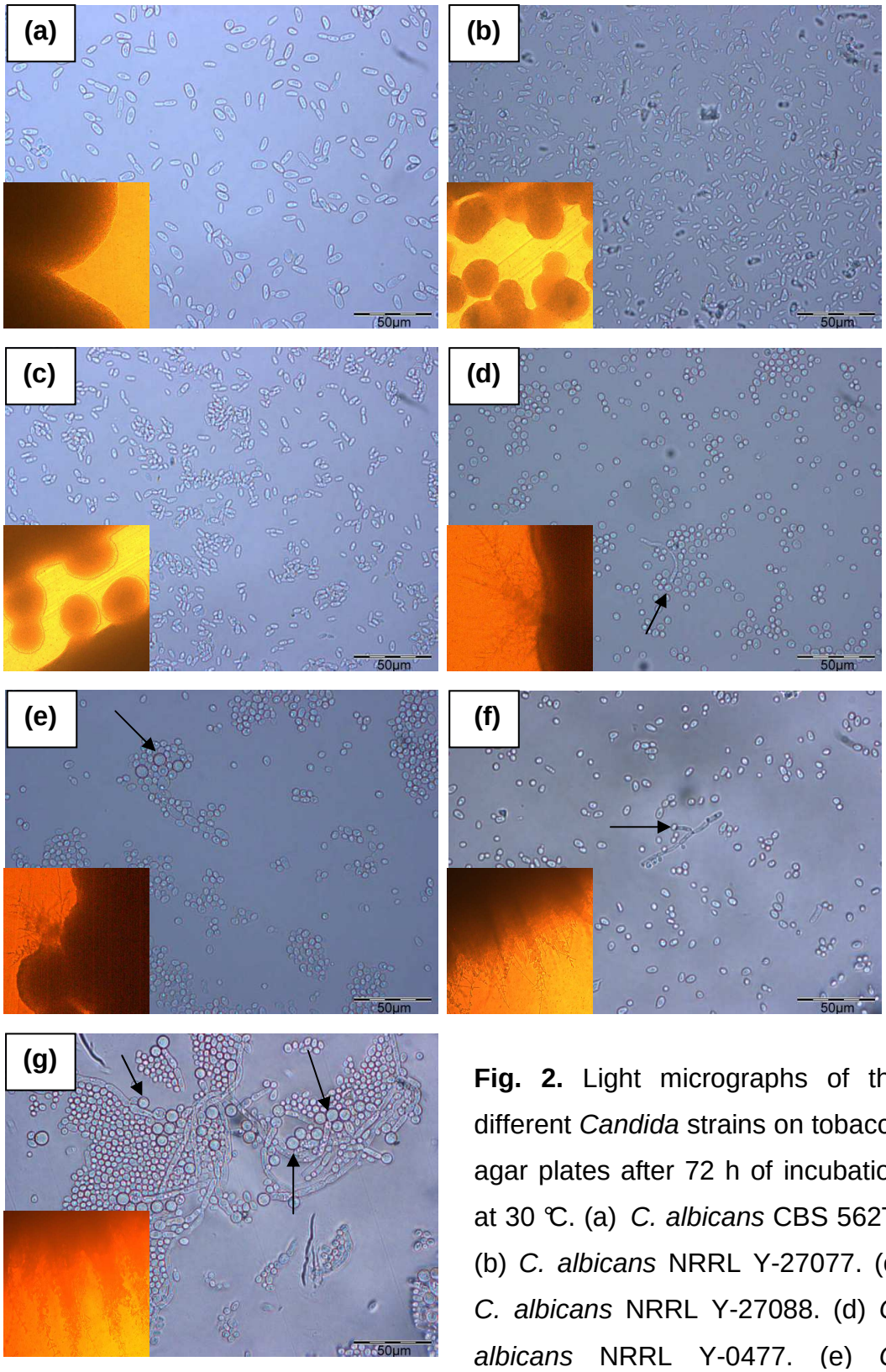


Fig. 2. Light micrographs of the different *Candida* strains on tobacco agar plates after 72 h of incubation at 30 ℃. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-27077. (c) *C. albicans* NRRL Y-27088. (d) *C. albicans* NRRL Y-0477. (e) *C. dubliniensis* NRRL Y-17841T. (f) *C. dubliniensis* NRRL Y-27787. (g) *C. dubliniensis* NRRL Y-27853. Inserts indicate presence or absence of hyphal fringes and arrows indicate chlamydospores.

2.4.3) Sunflower seed husk agar plates

Sunflower seed husk agar, also known as Pal's agar, was initially used for the identification of *Cryptococcus neoformans*, but later developed into a medium for the differentiation of *C. albicans* and *C. dubliniensis* (Mosaid *et al*., 2003). These authors used the whole sunflower seed for the preparation of the medium, but Khan *et al*. (2005) used only the husk of the seed. This was done in order to eliminate the interference of oil droplets (from seed extract) when examining the sample microscopically. Differentiation on this medium is also based on the production of hyphal fringes and chlamydospores by one species and not by the other species. The partial anoxic condition, due to the Dalmau technique, is one factor that is considered important for the production of chlamydospores by *Candida* species (Hayes, 1966).

On this medium the same characteristics were observed as with the tobacco agar. Most *C. albicans* strains tested (i.e. *C. albicans* CBS 562T, *C. albicans* NRRL Y-27077 and *C. albicans* NRRL Y-27088) produced smooth colonies, no hyphal fringes or chlamydospores (Fig. 3a, b, c). However, variation between strains were again observed with *C. albicans* NRRL Y-0477 (Fig. 3d) showing the same characteristics as all the *C. dubliniensis* strains used (i.e. *C. dubliniensis* NRRL Y-17841T, *C. dubliniensis* NRRL Y-27787 and *C. dubliniensis* NRRL-27853), that is hyphal fringe as well as chlamydospore production (Fig. 3e, f, g).

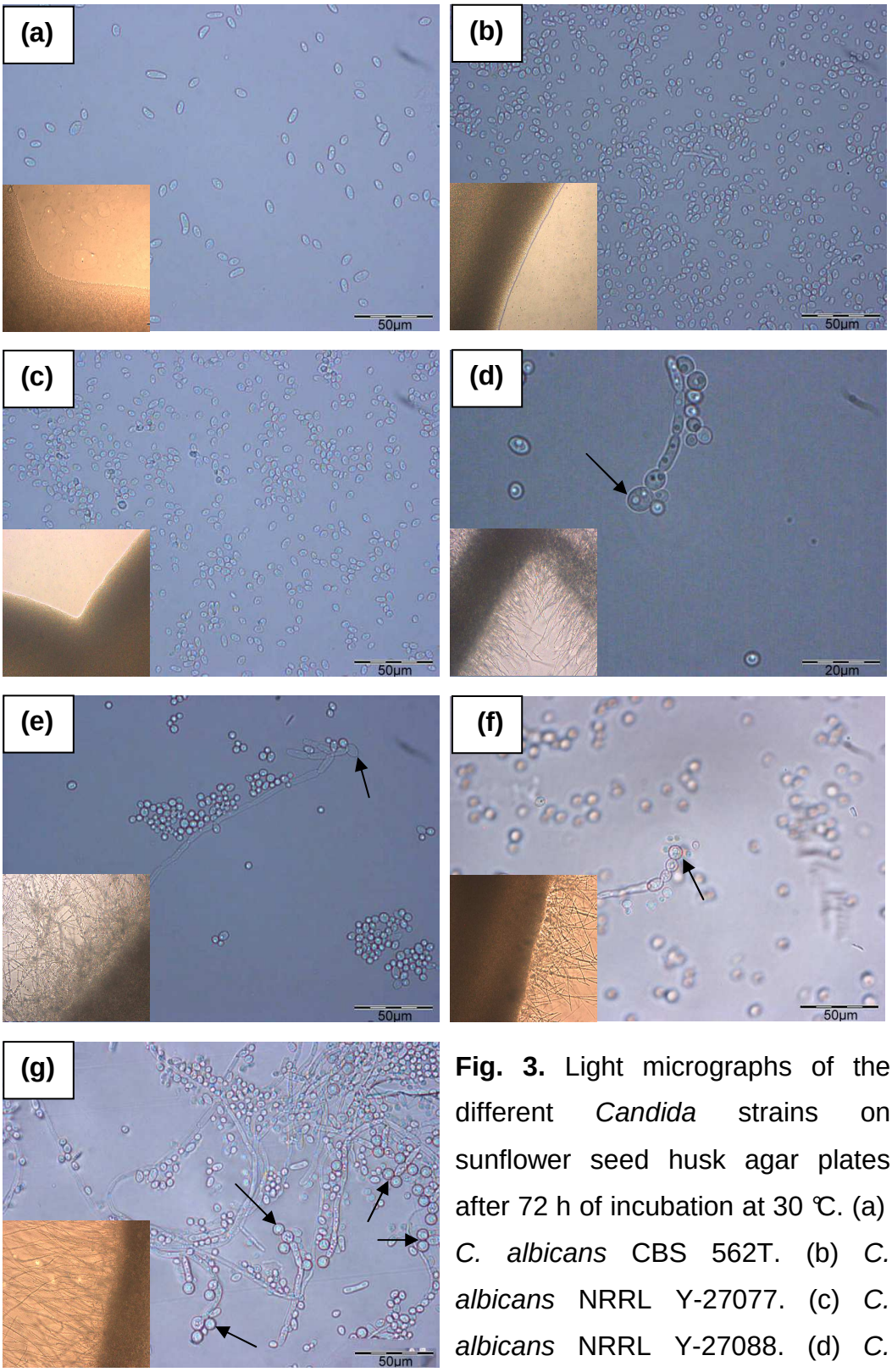


Fig. 3. Light micrographs of the different *Candida* strains on sunflower seed husk agar plates after 72 h of incubation at 30 ℃. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-27077. (c) *C. albicans* NRRL Y-27088. (d) *C. albicans* NRRL Y-0477. (e) *C. dubliniensis* NRRL Y-17841T. (f) *C. dubliniensis* NRRL Y-27787. (g) *C. dubliniensis* NRRL Y-27853. Inserts indicate presence or absence of hyphal fringes and arrows indicate chlamydospores.

2.4.4) Germ tube test

The initial stage during morphogenic conversion of dimorphic *Candida* species is the formation of germ tubes from yeast cells, leading to the development of pseudohyphae or true hyphae. This is believed to play a role in pathogenicity (Hudson *et al*., 2004). Germ tubes are defined as a cell with a rounded outgrowth with a length greater than or equal to the diameter of the parent cell (Vardar-Ünlü, 1998). Germ tube production is used to differentiate *C. albicans* and *C. dubliniensis* from other *Candida* species, since only these two *Candida* species have the ability to produce germ tubes (Sullivan *et al*., 1995). Germ tubes develop quickly and this makes it possible to rapidly identify these species. Germ tube formation can be induced by several substances, for example serum, egg white (albumin), *N*-acetylglucosamine, proline, ethanol (Pollack & Hashimoto, 1985) and peptone (Nakamoto, 1998).

Bovine serum albumin was found to be a potent inducer of germ tube formation by *Candida* strains in a short period, as quickly as 40 min, and is widely used to differentiate *C. albicans* and *C. dubliniensis* from other *Candida* species (Elmer *et al*., 1992). This was also found for the different strains used in this study. It was found that all the *Candida* strains tested have the ability to form germ tubes (Fig. 4) but that they differ in the time needed to form germ tubes and the number of germ tubes formed during that period (Table 2). It should also be noted that all the *C. dubliniensis* strains tested started to develop germ tubes more rapidly (from 30 min) than the *C. albicans* strains (from 45 min), except for *C. albicans* NRRL Y-0477. We can conclude that the *C. dubliniensis* strains had a better ability to produce germ tubes compared to the *C. albicans* strains under the same growth conditions or that serum is a better inducer of germ tubes in *C. dubliniensis* strains. Strain variation also exists since *C. albicans* NRRL Y-0477 produced germ tubes after only 30 min but *C. albicans* CBS 562T only produced germ tubes after 2 h.

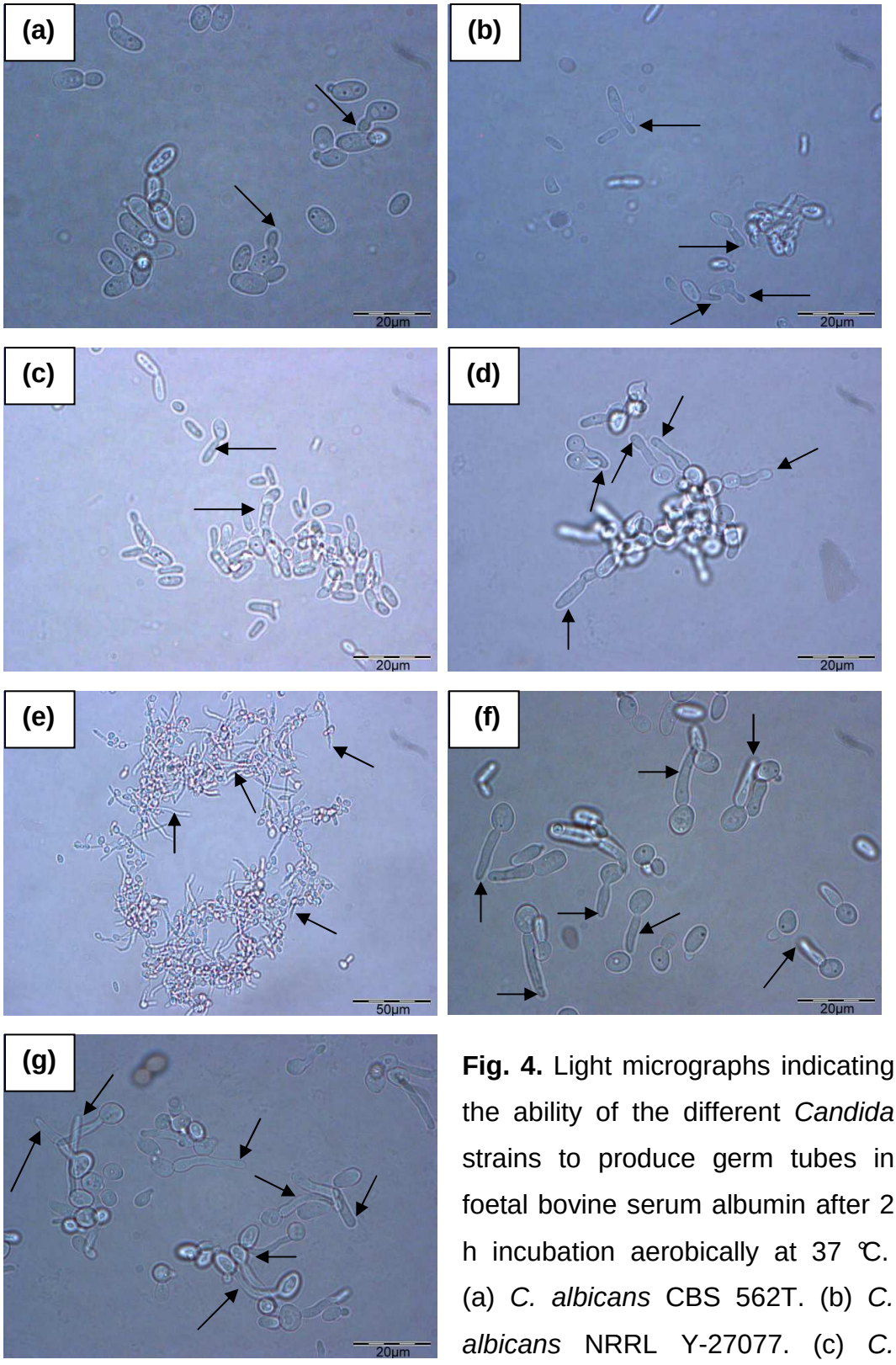


Fig. 4. Light micrographs indicating the ability of the different *Candida* strains to produce germ tubes in foetal bovine serum albumin after 2 h incubation aerobically at 37 ℃. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-27077. (c) *C. albicans* NRRL Y-27088. (d) *C. albicans* NRRL Y-0477. (e) *C. dubliniensis* NRRL Y-17841T. (f) *C. dubliniensis* NRRL Y-27787. (g) *C. dubliniensis* NRRL Y-27853. Arrows indicate germ tubes.

Table 2: Germ tube production by the different *Candida* strains after incubation in foetal bovine serum albumin.

	Germ tube production*			
Strain	**30 min**	**45 min**	**60 min**	**120 min**
C. albicans CBS 562T	-	-	-	++
C. albicans NRRL Y-27077	-	+	++	+++
C. albicans NRRL Y-27088	-	+	+	++
C. albicans NRRL Y-0477	+	+	++	+++
C. dubliniensis NRRL Y-17841T	+	++	+++	++++
C. dubliniensis NRRL Y-27787	+	+	++	++++
C. dubliniensis NRRL Y-27853	+	++	+++	++++

*(-) → no germ tubes; (++) → 20-40%; (+++) → 40-60%; (++++) → 60-80%; (+++++) → 80-100%

2.4.5) Growth at an elevated temperature

From the results obtained it is clear that none of the standard methods are reliable for differentiation of *C. albicans* from *C. dubliniensis* strains and that they should be used in conjunction with other identification techniques. Pinjon *et al*. (1998) experienced similar problems and decided to incubate the *Candida* isolates at temperatures between 42-45 ℃. Both *C. albicans* and *C. dubliniensis* grew to the same extent at 42 ℃. However, they found that, after 48 h of incubation at 45 ℃ on Emmons' modified Sabouraud glucose agar, none of the 120 *C. dubliniensis* isolates tested grew and that 97 of the 98 *C. albicans* isolates tested grew.

The results obtained in this study after 48 h incubation on YM agar plates at 45 ℃ are similar. From Fig. 5 it can be seen that *C. albicans* strains (Fig. 5a, b, c, d) have the ability to grow to the same extent at 45 ℃ and that *C. dubliniensis* strains (Fig. 5e, f, g) do not have the ability to grow at this temperature.

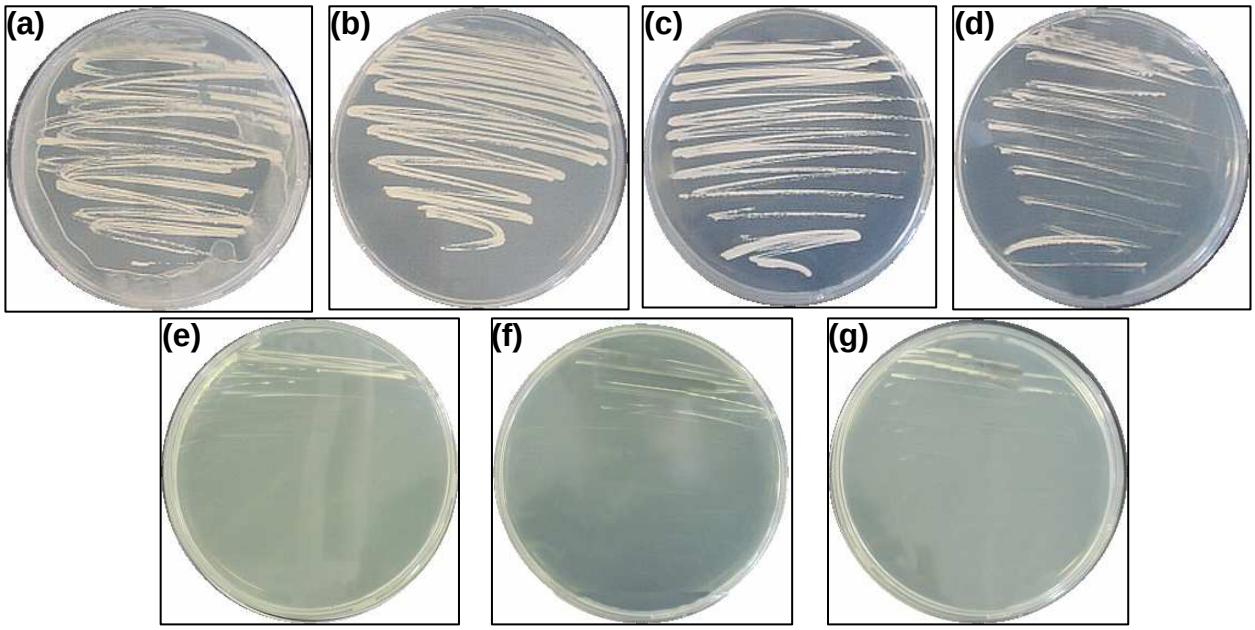


Fig. 5. Yeast malt extract agar plates indicating the growth of *Candida albicans* strains at 45 ℃ and the inability of *C. dubliniensis* strains to grow at 45 ℃. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-27077. (c) *C. albicans* NRRL Y-27088. (d) *C. albicans* NRRL Y-0477. (e) *C. dubliniensis* NRRL Y-17841T. (f) *C. dubliniensis* NRRL Y-27787. (g) *C. dubliniensis* NRRL Y-27853.

2.4.6) Lipase and phospholipase activity

Lipase and phospholipase activity are considered important virulence factors in pathogenic organisms. These enzymes damage and digest the cell membranes of the host which consist of proteins and lipids and this assists the invasion of the organism into the host tissue (mostly buccal and vaginal tissues) and also provides degraded materials as nutrients to the organism (Fotedar & Al-Hedaithy, 2005; Ogawa *et al*., 1992). Price *et al*. (1982) developed a plate assay for detecting phospholipase activity in *C. albicans*. This medium contained egg yolk or lecithin which is degraded by the phospholipase produced by *C. albicans*. The hydrolysis of the lipid substrates (phosphatidylcholine) in the egg-yolk leads to the formation of a calcium complex with fatty acids and it is then analysed by determining the precipitation zones (Pz values) around the colonies on the medium. This method is very useful for screening a large number of samples for

phospholipase activity. The Pz values indicated in Table 3 illustrates the ratio of the colony diameter to the diameter of the colony-plus-clearance zone (lipase production) or the colony-plus-precipitation zone (phospholipase production) (Price *et al*., 1982). Low Pz values indicate higher enzyme activity and high Pz values indicate a lower enzyme activity.

Extracellular lipase production has been observed in *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* with the highest lipase production detected in *C. albicans* with the use of a lipase assay kit (Bramono *et al*., 2006). Lipase production and its role in virulence in *Candida* species, especially in *C. dubliniensis*, have not received much attention. However, Slifkin (2000) found with the Tween 80 opacity test that only *C. albicans* and not *C. dubliniensis* has lipolytic activity. Vidotto *et al*. (2004) indicated by the use of a semi-quantitative API-ZYM system that both *C. albicans* and *C. dubliniensis* produced lipases but at low nano molar level. In this study it was found that the *C. albicans* strains tested had a higher lipase activity (Pz values: 0.4 ± 0.1 to 0.7 ± 0.1) when compared to the *C. dubliniensis* strains (Pz values: 0.7 ± 0.1 to 0.9 ± 0.0), after ten days of incubation on tributyrate agar (Table 3). As can be seen there is considerable variation between strains.

The results obtained for the phospholipase activity also indicated that *C. albicans* strains tested have a higher phospholipase activity (0.4 ± 0.0 to 0.6 ± 0.1) than the *C. dubliniensis* strains tested (0.5 ± 0.1 to 0.9 ± 0.1) after seven days of incubation on the phospholipase egg yolk medium (Table 3). Here variation between strains was also observed. Hannula *et al*. (2000) also found that *C. albicans* produced phospholipases in greater amounts compared to *C. dubliniensis*. However, Vidotto *et al*. (2004) found that the 26 *C. dubliniensis* isolates tested (Pz values: 0.96-0.75) and the 27 *C. albicans* isolates tested (Pz values: 0.86-0.79), which were isolated from the oral cavity of patients with HIV, have the same ability to produce phosholipases after two to nine days of incubation at 37 ℃. In addition, Fotedar & Al-Heda ithy (2005) found that 52 *C. albicans* isolates, from HIV negative patients, had the ability to produce phospholipases (Pz values: 0.37-0.74) after seven days of incubation at 37 ℃ and that none of the 87 *C. dubliniensis* isolates, also from HIV negative patients, produced phospholipases. It was also indicated that isolates obtained

from infected individuals are capable of higher phospholipase production compared to isolates from healthy individuals indicating the importance of the origin of the isolates (i.e. isolates from healthy individuals or from patients with candidiasis) (Borst & Fluit, 2003). These factors may influence the production and activity of these enzymes and emphasise the importance of enzyme production during pathogenicity.

The higher lipase and phospholipase activities of *C. albicans* may indicate why this species is more successful as a pathogen compared to *C. dubliniensis*. It is interesting to note that some *Candida* strains have low lipase activity (Pz values: 0.9 ± 0.0) but high phospholipase activity (Pz values: 0.5 ± 0.1) in the case of *C. dubliniensis* NRRL Y-27787. The opposite was also observed for *C. dubliniensis* NRRL Y-27853 (lipase, Pz values: 0.7 ± 0.1; phospholipase, Pz values: 0.9 ± 0.0). This phenomenon might enable strains to colonise different substrates or tissues or to adapt to different environments, therefore leading to different types of infections such as oral, vaginal or systemic candidiasis.

Table 3: Pz values for the lipase and phospholipase activity of the different *Candida* strains.

Strains	**Lipase activity**	**Phospholipase activity**
	Pz value*	**Pz value***
	10 days	**7 days**
C. albicans CBS 562T	0.4 ± 0.1	0.4 ± 0.0
C. albicans NRRL Y-0477	0.7 ± 0.1	0.6 ± 0.1
C. albicans NRRL Y-27077	0.6 ± 0.1	0.5 ± 0.0
C. albicans NRRL Y-27088	0.5 ± 0.1	0.5 ± 0.0
C. dubliniensis NRRL Y-17841T	0.8 ± 0.1	0.9 ± 0.1
C. dubliniensis NRRL Y-27787	0.9 ± 0.0	0.5 ± 0.1
C. dubliniensis NRRL Y- 27853	0.7 ± 0.1	0.9 ± 0.0

*Ratio of colony diameter to diameter of colony-plus-clearance zone / colony-plus-precipitation zone.

2.4.7) Hydrophobic microsphere assay

Hydrophobicity is also seen as a very important virulence factor since it is known to be associated with the attachment of the pathogen to the host cell surface (Gutierrez *et al*., 2002). Several methods have been developed and are used to quantify microbial hydrophobicity. This includes hydrophobic interaction chromatography (Jones *et al*., 1996), hydrophobic microsphere assay (HMA) (Hazen & Hazen, 1987), microbial adhesion to hydrocarbons (Rodrigues *et al*., 1999; Rosenberg, 2006), co-aggregation with *Fusobacterium nucleatum* (Jabra-Rizk *et al*., 2001), measurement of contact angles between microbial lawns and a polar liquid (e.g. water, formamide, methylene iodide and/or α-bromonaphthalene) (Henriques *et al*., 2002; van der Mei *et al*., 1998) and flow cytometry (Colling *et al*., 2005). The most common method, which only gives qualitative information, is HMA (Henriques, 2004). However, it has been stated that with this method cell surface hydrophobicity is masked, due to the presence of other forces such as electrostatic forces that might interfere with the interactions (Aguedo *et al*., 2003; Doyle, 2000).

From the results obtained with HMA (Fig. 6), it was found that all the *Candida* strains tested were hydrophobic at both 25 ℃ and 3 7 ℃ (Table 4). However, all the *C. dubliniensis* strains were more hydrophobic at 37 ℃ and the *C. albicans* strains more hydrophobic at 25 ℃. Jabra-Rizk *et al*. (2001) also found that both *C. albicans* and *C. dubliniensis* were hydrophobic at 25 ℃, but that only *C. dubliniensis* were hydrophobic at 37 ℃. *Candida albicans* NRRL Y-0477 and *C. dubliniensis* NRRL Y-17841T showed the highest percentage hydrophobicity at 25 ℃ and 37 ℃ compared to the o ther *C. albicans* and *C. dubliniensis* strains respectively.

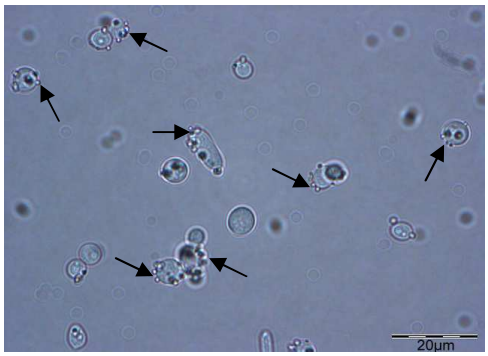


Fig. 6. Light micrograph indicating the attachment of the microspheres to *Candida dubliniensis* NRRL Y-17841T cells (1000X). Arrows indicate microspheres attached to the cells.

Table 4: The percentage hydrophobicity of the different *Candida* strains.

Strains	% Hydrophobicity*	
	25 ℃	**37 ℃**
C. albicans CBS 562T	20.8	15.0
C. albicans NRRL Y-27077	22.2	10.8
C. albicans NRRL Y-27088	46.2	11.9
C. albicans NRRL Y-0477	68.5	21.1
C. dubliniensis NRRL Y-17841T	70.0	83.1
C. dubliniensis NRRL Y-27787	28.2	58.6
C. dubliniensis NRRL Y-27853	34.8	49.5

*Percentage colony forming units having three or more microspheres attached.

2.4.8) Growth of planktonic cells

The different growth phases of organisms have been identified as the initial lag phase, followed by the exponential growth phase, where the cells grow rapidly by using the available carbon source in the medium (e.g. glucose) (Werner-Washburne *et al*., 1993). When the primary available carbon source becomes depleted, some organisms undergo a shift in metabolism, a diauxic shift, and use an alternative carbon source in the medium (e.g. ethanol), this was also found in *C. albicans* which uses cellular lipids or medium peptides for further

growth (Uppuluri & Chaffin, 2007). This phase is then followed by the stationary phase when all the carbon sources become depleted and cell growth slows with no further net increase in cell number. In this phase, organisms can remain viable for several days and they have the ability to resume growing when nutrients are available again, making this phase advantageous for some organisms (Uppuluri *et al*., 2006; Werner-Washburne *et al*., 1993). In *C. albicans* it was found that stationary phase cells were more adherent to host tissues with the production of more true hyphae and higher resistance to a wide range of antifungals (e.g. azole drugs) as well as oxidative stress (Beggs, 1984; King *et al*., 1980; Lyons & White, 2000; Westwater *et al*., 2005). One aspect of the growth phases of *C. albicans* that is still poorly understood is the timing of entry into the different phases, especially the stationary phase. Some authors accept 24 h to 48 h grown cultures as being in the stationary phase whereas others stated that stationary phase starts between three and eight days (Song *et al*., 2004; Zhao *et al*., 2005).

The results obtained for the growth of planktonic cells (Fig. 7) indicated that all the *Candida* strains grew to the same extent when grown under the same conditions. After 2 h of incubation at 37 ℃ the la g phase was overcome and for the next 6 h the strains grew exponentially utilising glucose as carbon source. After 8 h of incubation there was a decrease in growth and no net increase in the optical density, probably due to glucose depletion.

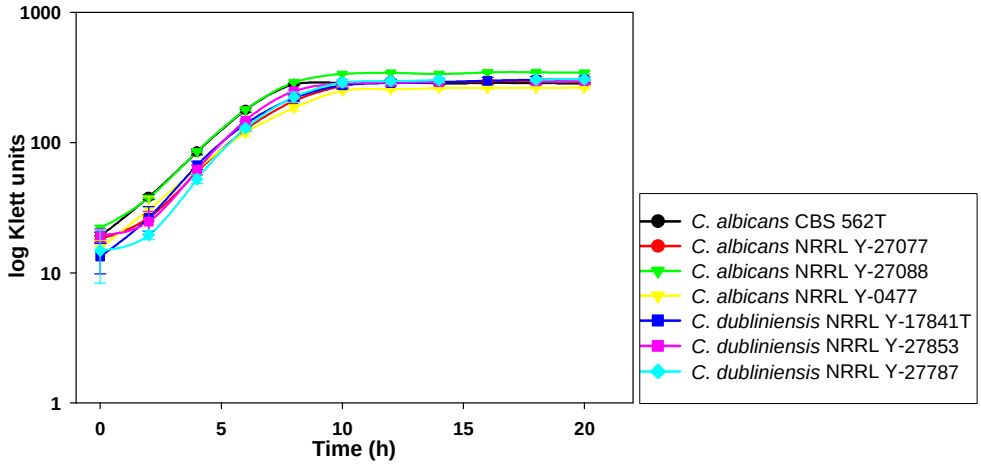


Fig. 7. Growth of planktonic *Candida* strains incubated at 37 ℃ for up to 20 h measured by a Klett-Summerson photoelectric colorimeter.

2.4.9) Biofilm production

From Fig. 8 it can be seen that all the *Candida* strains have the ability to produce mature biofilms at 37 ℃ in microtiter plat es. However, they differ in the amount of biofilm produced by each strain, with *C. albicans* NRRL Y-27088 producing significantly less biofilm than the other strains. From this it is also clear that there is variation between strains of both species in their ability to produce biofilms. This agrees with the results of Henriques *et al*. (2006) when they evaluated biofilm formation by *C. albicans* and *C. dubliniensis* using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay as well as crystal violet staining. The graph (Fig. 8) indicates that, for the first 24 h the yeast biofilm increased for most of the strains tested, but not for *C. albicans* NRRL Y-27088. After 48 h some of the strains (i.e. *C. albicans* CBS 562T, *C. albicans* NRRL Y-0477, *C. albicans* NRRL Y-27077 and *C. albicans* NRRL Y-27088) exhibited a decrease in the OD and this might be due to the phenomenon that slow growing cells have a decreased cell size (Chaffin, 1984). Interestingly this was only found for the *C. albicans* strains tested and not for the *C. dubliniensis* strains tested. This might indicate that *C. dubliniensis* have a better ability to produce biofilms when nutrients are limited.

According to Uppuluri *et al*. (2006) the structural complexity of a biofilm might result in an environment where cells might have different physiological phases, i.e. when you compare cells at the bottom of the biofilm where nutrients are limited to the fast growing cells at the top of the biofilm where nutrients are still available. This makes it difficult to accurately identify the different growth phases of cells in a biofilm.

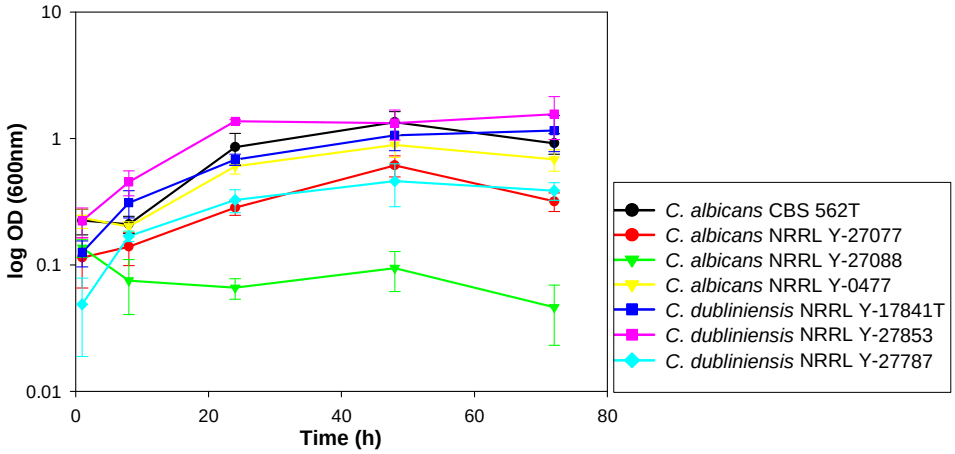


Fig. 8. Biofilms produced by *Candida* strains in 96 well microtiter plates at 37 ℃ for up to 72 h and quantified in terms of biomas s.

2.4.10) D1/D2 Sequencing

From the phenotypic results it became clear that *C. albicans* NRRL Y-0477 was phenotypically similar to the examined *C. dubliniensis* strains. To confirm that this strain was in fact *C. albicans*, sequencing of the D1/D2 domain of the variable region of the large ribosomal DNA subunit (26S rDNA), which is approximately 600 bases in size, was performed (Kurtzman & Robnett, 1998). The results confirmed that *C. albicans* NRRL Y-0477 was in fact a *C. albicans* strain.

2.5) Conclusions

There are several techniques available for the identification, differentiation and characterisation of *Candida* species. From the methods that have been used in this study it was found that there is phenotypic variation within species. A good example in this study is *C. albicans* NRRL Y-0477, which resembled *C. dubliniensis* with almost every method used. Only growth at 45 °C and D1/D2 sequencing could clearly distinguish between these species.

Following this characterisation study it was decided to use three phenotypically distinct strains for further studies i.e. *C. albicans* CBS 562T, *C. albicans* NRRL Y-0477 and *C. dubliniensis* NRRL Y-17841T.

Arachidonic acid (20:4) plays an important role during *C. albicans* infection, but there are no definitive results on the effect of 20:4 on growth and

morphogenesis in *C. albicans*. In addition, the effect of 20:4 on growth and morphology of *C. dubliniensis* is unknown. This will be addressed in the next chapter.

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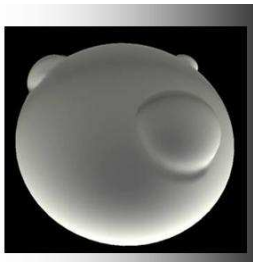
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CHAPTER 3

Effect of arachidonic acid on growth and biofilm formation by selected *Candida* strains

3.1) Abstract

Biofilm formation plays an important role during infection by pathogenic organisms, due to the protective environment it provides for the pathogen. In addition, biofilms have an increased resistance to antimicrobial agents and the host's immune defences. The two closely related *Candida* species, *C. albicans* and *C. dubliniensis*, are capable of biofilm formation and are increasing as causative agents of biofilm infections in especially immunocompromised patients. Arachidonic acid (20:4), derived from the infected host cells, is important during *C. albicans* infection, serving as carbon source and for the production of 20:4 metabolites, which serve as virulence or morphogenic factors. Conflicting results are presented in literature regarding the effect of 20:4 on the morphological changes in *C. albicans*. One such report indicated that 20:4 has no effect on morphogenesis (the conversion of yeast cells to hyphal stages) in *C. albicans*, while another found an inhibiting effect. In addition, the effect of 20:4 on growth and morphology of *C. dubliniensis* is unknown. Therefore the aim of this study was to evaluate the effect of 20:4 on growth and biofilm formation by the selected *C. albicans* and *C. dubliniensis* strains. The methods that were used in this study include determination of planktonic growth using a Klett-Summerson photoelectric colorimeter, the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), to measure metabolic activity of planktonic cells and biofilms. Light microscopy of biofilms grown in chamber slides and scanning electron microscopy of biofilms grown on silicone disks were used to evaluate the morphology of cells in a biofilm. The viability of the cells in a biofilm was evaluated by confocal laser scanning microscopy and the use of the LIVE/DEAD® *Bac*light™ Bacterial Viability kit. The results obtained indicated that 1 mM 20:4 has little to no effect on the growth and metabolic activity of planktonic cells and biofilms, as well as on the morphology and viability of the cells in the biofilms of the selected strains.

3.2) Introduction

Many microbes, including yeasts, are found in their natural habitats as biofilms that are attached to surfaces, and not as free-floating organisms (Ramage *et al*., 2005). It is believed that the reasons for biofilm formation include

increased availability of nutrients, metabolic support, gaining of new genetic characteristics and to be protected from the environment (Branda & Kolter, 2004). It is known that yeast biofilms have increased resistance to antifungal treatment and a greater competence against host immune defences than the planktonic unicellular microbes (Jabra-Rizk *et al*., 2004). As a consequence of this, biofilms are difficult to remove and are a source of many uncontrollable infections. Therefore, biofilms are getting progressively distinguished as an important health problem for patients with microbial infections.

As we saw from the previous chapter, the closely related dimorphic yeasts, *C. albicans* and *C. dubliniensis*, are associated with biofilm infections, causing superficial and invasive diseases (Ramage *et al*., 2001a; Xiaogang *et al*., 2003). These yeasts can survive as yeast cells (thick-walled chlamydospores or blastospores), pseudohyphae or hyphae, depending on the growth conditions (Ramage *et al*., 2001b). Fungal infections are believed to be greatly influenced by the hyphal form (Chandra *et al*., 2001).

Candida albicans infection is associated with the release of the bioactive molecule, 20:4, from the infected host cell membrane (Brash, 2001; Deva *et al*., 2001). Deva *et al*. (2000) found that *C. albicans* can grow on low micromolar concentrations of exogenously fed 20:4 as sole carbon source. In addition, 20:4 is known to be an important modulator of the immune response of host cells through the production of eicosanoids (Noverr *et al*., 2003). The presence of four double bonds in 20:4, allows it to react easily with oxygen either, enzymatically (oxygenases) or non-enzymatically (oxidative stress) leading to the production of eicosanoids, including prostaglandins, leukotrienes and other bioactive metabolites (Brash, 2001). These compounds are known to be important during infection by affecting the host's defences, including immune responses, enhancing vascular permeability and facilitating the invasion of the host tissue/cells (Chen *et al*., 2000; Deva *et al*., 2001). It may be speculated that changes in these *Candida* species, related to the presence of 20:4, could aid in pathogenicity.

Therefore, the aim became to study the effect of exogenously fed 20:4 on growth and biofilm formation by the selected *C. albicans* and *C. dubliniensis* strains.

3.3) Materials and methods

3.3.1) Strains used

The following strains were used in this study: *Candida albicans* CBS 562T, *C. albicans* NRRL Y-0477, *C. dubliniensis* NRRL Y-17841T. All strains were maintained on yeast malt extract (YM) agar [10 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone, 16 g agar, 1000 mL distilled water (dH_20)] at room temperature.

3.3.2) Effect of 20:4 on planktonic growth

The *Candida* strains were grown on YM agar plates for 24 h at room temperature. The strains were then inoculated into 200 mL sterile yeast nitrogen base (YNB) broth [6.7 g YNB, 1000 mL dH_20] containing 10 $g.L^{-1}$ glucose (pre-inoculum) in 1000 mL conical flasks and incubated aerobically at 37 ℃ for 12 h. The cells were diluted to 10 Klett units on a Klett-Summerson photoelectric colorimeter in 100 mL YNB containing either 10 $g.L^{-1}$ glucose or 1 mM 20:4 (30.5 $g.L^{-1}$) (Sigma-Aldrich, USA) (in ethanol) as carbon source in 500 mL conical flasks and incubated aerobically at 37 ℃. The optical density (OD) of the cultures was measured in Klett units every 2 h for 20 h. Growth curves were constructed from the obtained data.

3.3.3) Effect of 20:4 on the metabolic activity of planktonic cells

The strains, from 24 h old cultures on YM agar plates, were grown in 20 mL sterile YNB broth containing 10 $g.L^{-1}$ glucose at 37 ℃ for 24 h in sterile centrifuge tubes. The cells were harvested by centrifugation for 5 min at 4000 *g*, washed with phosphate buffered saline (PBS) (OXOID, UK), and diluted to 1 x 10^6 $cells.mL^{-1}$ in 20 mL sterile YNB broth without glucose. Arachidonic acid was added to a final concentration of 1 mM. Appropriate controls were included. The cells were incubated at 37 ℃ and har vested (5 min at 4000 *g*) after 6 h and 12 h, washed and resuspended in 5 mL sterile YNB broth without glucose. The cells were counted with a hemacytometer and diluted to 5 x 10^6 $cells.mL^{-1}$. Hundred microlitres from each time point was dispensed into the wells of a 96-well microtiter plate (Corning Incorporated, Costar®, USA) with appropriate controls. To each well 50 µL of 2,3-bis(2-methoxy-4-nitro-5-

sulfophenyl)-5[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) (Sigma Chemicals, USA) (0.5 g XTT in 1000 mL Ringer's lactate solution, filter sterilised, aliquoted and stored at -70 ℃) and 4 µL of 1 mM menadione (Fluka, USA) (10 mM menadione in acetone, further diluted to 1 mM) were added and incubated for 3 h in the dark at 37 ℃. The OD was then read at 492 nm on a Labsystems iEMS Reader (Thermo Bioanalysis, Helsinki, Finland) (Bachmann *et al*., 2002).

3.3.4) Effect of 20:4 on morphology and viability of biofilms

a) Light microscopy

The strains, from 24 h old cultures on YM agar plates, were inoculated into 5 mL sterile YNB broth containing 10 $g.L^{-1}$ glucose in sterile centrifuge tubes and incubated at 30 ℃ for 48 h. The cells were harvested by centrifugation for 10 min at 4000 *g*, washed and resuspended into filter sterilised RPMI-1640 medium (Sigma-Aldrich, USA). The cells were then counted and diluted to 1 x 10^6 $cells.mL^{-1}$ in 10 mL filter sterilised RPMI-1640 medium. Three millilitres of this suspension were pippeted into chamber slides (Lab-Tek® Chamber Slide™ System, Naperville). Appropriate controls were included. The slides were incubated at 37 ℃ for 72 h to allow biofilm formation (Ramage *et al*., 2001b). The biofilms were then washed carefully with PBS and examined with a light microscope (Zeiss Axioplan, Colorview soft imaging system, West Germany).

b) Scanning electron microscopy

Biofilms of the *Candida* strains were prepared as above, the only difference being that the biofilms were grown on silicone disks (6 mm in diameter) in chamber slides. The slides were incubated at 37 ℃ for 48 h to allow biofilm formation on the silicone disks (Hawser & Douglas, 1994). The disks were then washed carefully with PBS. The biofilms on the disks were fixed with 3 % (v/v) gluterdialdehyde (primary fixative) (Merck, Darmstadt, Germany) in phosphate buffer (pH 7.0) overnight at room temperature. After washing the disks carefully with phosphate buffer, the biofilms were fixed with 0.5 % (v/v) osmiumtetroxide (secondary fixative) (Merck, Darmstadt, Germany) in phosphate buffer (pH 7.0) for 2 h at room temperature. The dehydration steps

were performed with different ethanol concentrations (50 %, 70 %, 95 %) for 15 min each and then finally twice with absolute ethanol for 60 min. The disks were washed again with phosphate buffer. After the biofilms had undergone critical point drying, the disks were mounted on metal stubs and coated with gold. The biofilms were then examined with a Shimadzu SSX-550 Superscan scanning electron microscope.

c) Confocal laser scanning microscopy

Biofilms of the *Candida* strains were prepared as above in chamber slides. The biofilms in the chamber slides were carefully washed with 0.9 % (w/v) NaCl. To evaluate the viability of the cells in the biofilm, the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit (Invitrogen, Molecular Probes, USA), which is composed of SYTO-9 and propidium iodide (PI), was used according to the manufacturer's instructions (Jin *et al*., 2005). 1,4 Diazabicyclo-[2,2,2]octane (DABCO) (Sigma-Aldrich, USA), which stabilises fluorescence, was added and the slides were examined with a confocal laser scanning microscope (CLSM) (Nikon TE 2000, Japan).

3.3.5) Effect of 20:4 on the metabolic activity of biofilms

The strains, from 24 h old cultures on YM agar plates, were grown in 20 mL sterile YNB broth, containing 10 $g.L^{-1}$ glucose, at 30 ℃ for 48 h in sterile centrifuge tubes. The cells were harvested by centrifugation for 10 min at 4000 *g*, washed with sterile PBS and resuspended in 10 mL filter sterilised RPMI-1640 medium. The cells were counted with a hemacytometer and diluted to 1 x 10^6 $cells.mL^{-1}$ in filter sterilised RPMI-1640 medium. Arachidonic acid was added to this suspension to a final concentration of 1 mM and 100 µL were dispensed into the wells of a 96-well microtiter plate and incubated at 37 ℃ for 48 h in order to allow biofilm formation (Ramage *et al*., 2001b). Appropriate controls were included. At different time intervals the wells were washed three times with sterile PBS, and the XTT assay performed as above.

3.3.6) Statistical analysis

All experiments were performed at least in triplicate. The *t*-test was performed to determine if the data sets obtained were significantly different from each other with a P-value equal to or less than 0.05.

3.4) Results and discussion

3.4.1) Effect of 20:4 on planktonic growth

Deva *et al*. (2000) found that *C. albicans* can utilise 20:4 as carbon source. However, they found growth inhibition at concentrations higher than 20 µM and maximum growth at 3 µM. Erb-Downward & Noverr (2007) found that 500 µM 20:4 in Sabouraud dextrose broth, did not affect the growth of planktonic *C. albicans* strains. In this study (Fig. 1), it was indicated that 1 mM 20:4 had a slight inhibiting effect on the growth of *C. albicans* CBS 562T (Fig. 1a) during the first 10 h of growth resulting in a longer lag phase and delayed entry into stationary phase (12 h instead of 8 h). The final cell density was however not affected. In the case of *C. albicans* NRRL Y-0477 (Fig. 1b) and *C. dubliniensis* NRRL Y-17841T (Fig. 1c) there was no significant inhibition of growth in the presence of 1 mM 20:4. This indicates that these *Candida* strains are able to grow equally well on 1 mM 20:4, dissolved in ethanol (28 millimole carbon) as on 10 $g.L^{-1}$ glucose (33 millimole carbon). These results differ from Deva *et al*. (2000) possibly due to strain differences or differences in culture conditions. They incubated 1 x 10^3 $cells.mL^{-1}$ for 24 h at 37 ℃ in an inorganic salt medium containing KH_2PO_4, $(NH_4)_2SO_4$, $CaCl_2$, $MgSO_4$ and biotin with different concentrations of 20:4 and determined growth by plating out serial dilutions of the samples onto Sabouraud agar plates and counting the colony forming units after 24 h of incubation.

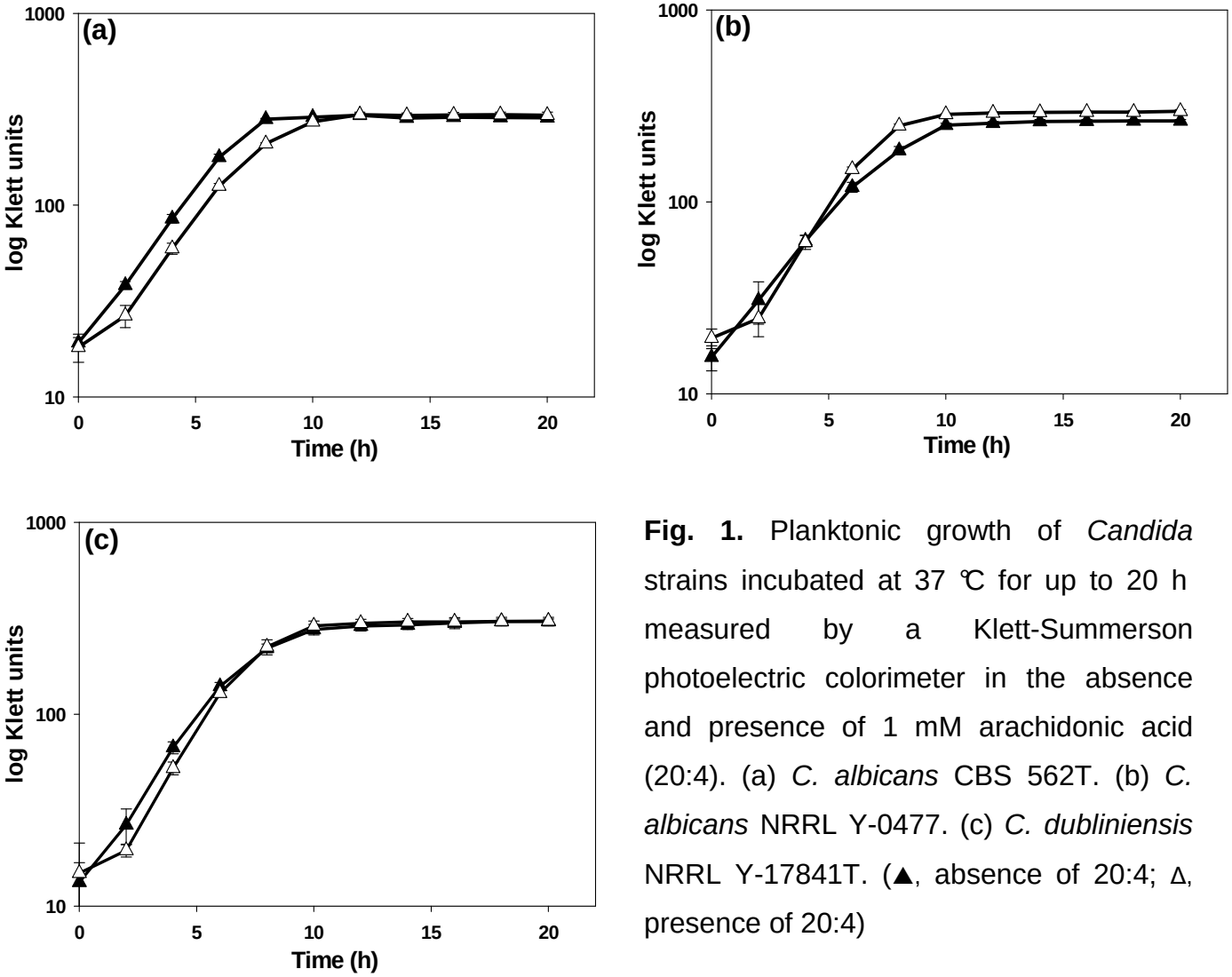


Fig. 1. Planktonic growth of *Candida* strains incubated at 37 ℃ for up to 20 h measured by a Klett-Summerson photoelectric colorimeter in the absence and presence of 1 mM arachidonic acid (20:4). (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-0477. (c) *C. dubliniensis* NRRL Y-17841T. (▲, absence of 20:4; ∆, presence of 20:4)

3.4.2) Effect of 20:4 on the metabolic activity of planktonic cells

The reduction of XTT is used to examine the viability of yeast cells by measuring the metabolic activity (Kuhn *et al*., 2003). The tetrazolium salt, XTT, is converted to a coloured formazan product, which is water soluble, diffuses easily out of the cells, and is easily measured in cellular supernatants in terms of OD at 492 nm. This conversion is the result of a metabolic activity by the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases. However, this assay has some limitations, including the fact that there is not a definite certainty of a relationship between the colorimetric signal and the cell number.

When the metabolic activity of planktonic cells of *C. albicans* CBS 562T (Fig. 2a) and *C. dubliniensis* NRRL Y-17841T (Fig. 2c) were measured, no

significant difference could be observed between 6 h and 12 h of growth. However, this was not the case with *C. albicans* NRRL Y-0477 (Fig. 2b), where it was found, contrary to expectations, that stationary phase (12 h) cells are metabolically more active than cells in exponential phase (6 h). It can also be seen that *C. albicans* NRRL Y-0477 (Fig. 2b) has a higher metabolic activity in the presence and absence of 20:4 compared to the other two strains (Fig. 2a, c). In addition, there is no significant difference in metabolic activity in the presence and absence of 20:4. This is true for all three strains tested. It can be concluded that 1 mM 20:4 has no effect on metabolic activity of planktonic *Candida* cells.

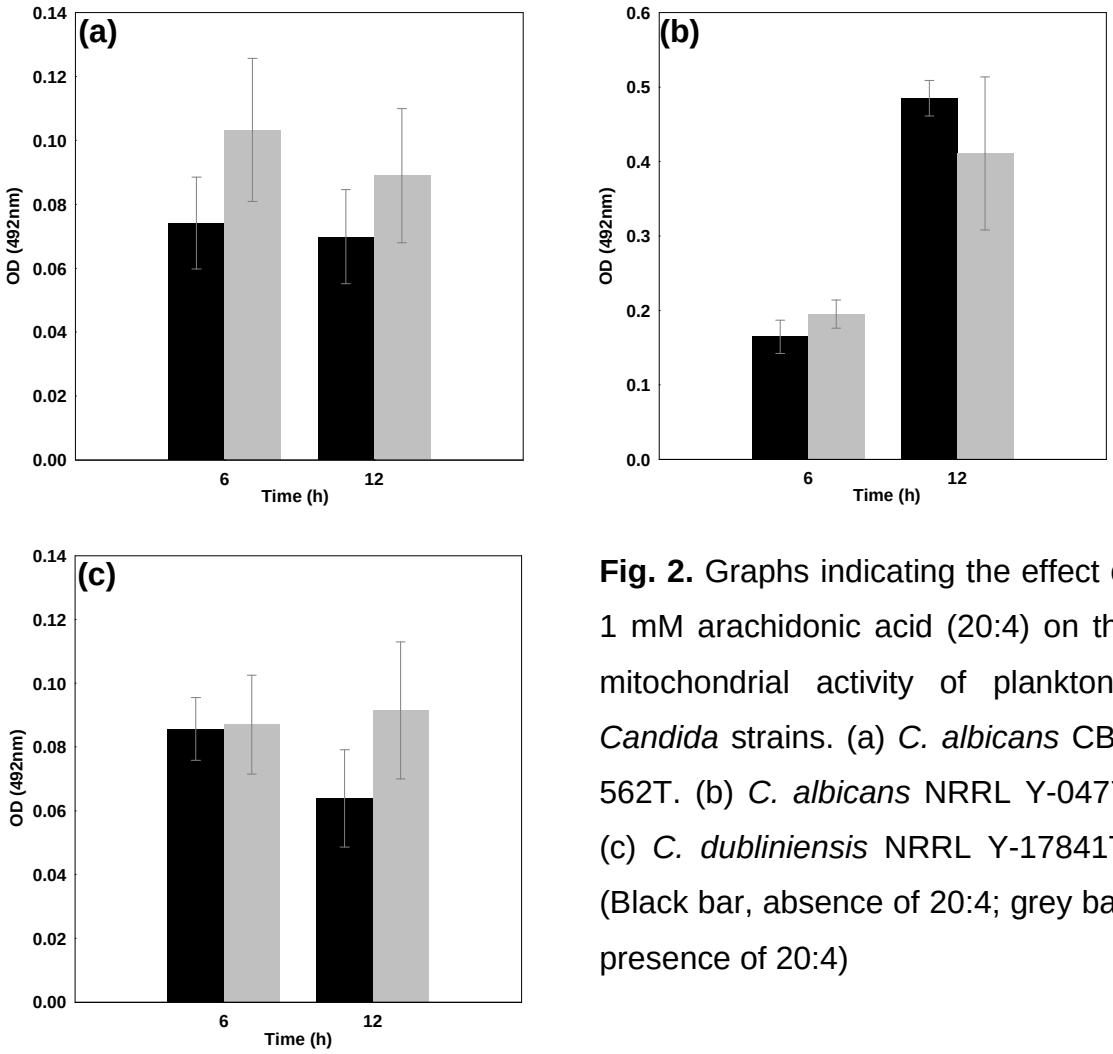


Fig. 2. Graphs indicating the effect of 1 mM arachidonic acid (20:4) on the mitochondrial activity of planktonic *Candida* strains. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-0477. (c) *C. dubliniensis* NRRL Y-17841T. (Black bar, absence of 20:4; grey bar, presence of 20:4)

3.4.3) Effect of 20:4 on morphology and viability of biofilms

a) Light microscopy and scanning electron microscopy

From the light micrographs of mature *Candida* biofilms formed on glass chamber slides (Fig. 3), it is clear that the biofilms consist of budding yeast cells, pseudohyphae or true hyphae or a combination of these morphologies. This combination of morphologies can be seen especially in *C. albicans* NRRL Y-0477 (Fig. 3c, d) and *C. dubliniensis* NRRL Y-17841T (Fig. 3e, f). Here again it can be seen that *C. albicans* NRRL Y-0477 resembles *C. dubliniensis* strains rather than *C. albicans* strains (as discussed in Chapter 2), whereas *C. albicans* CBS 562T (Fig. 3a) only formed yeast cells. When the biofilms grown in the absence and presence of 1 mM 20:4 are compared, it is clear that there is no effect on the morphology.

The same observations were found using scanning electron microscopy (Fig. 4), even though the biofilms were grown on silicone disks. In contrast to our results, Clément *et al*. (2007) found that 20:4, isolated from bovine whey cream, inhibits the conversion from yeast to hyphal stages (morphogenesis) in *C. albicans*. However, Noverr & Huffnagle (2004) found that 20:4, as well as other long chain fatty acids, does not have an effect on morphogenesis of *C. albicans*.

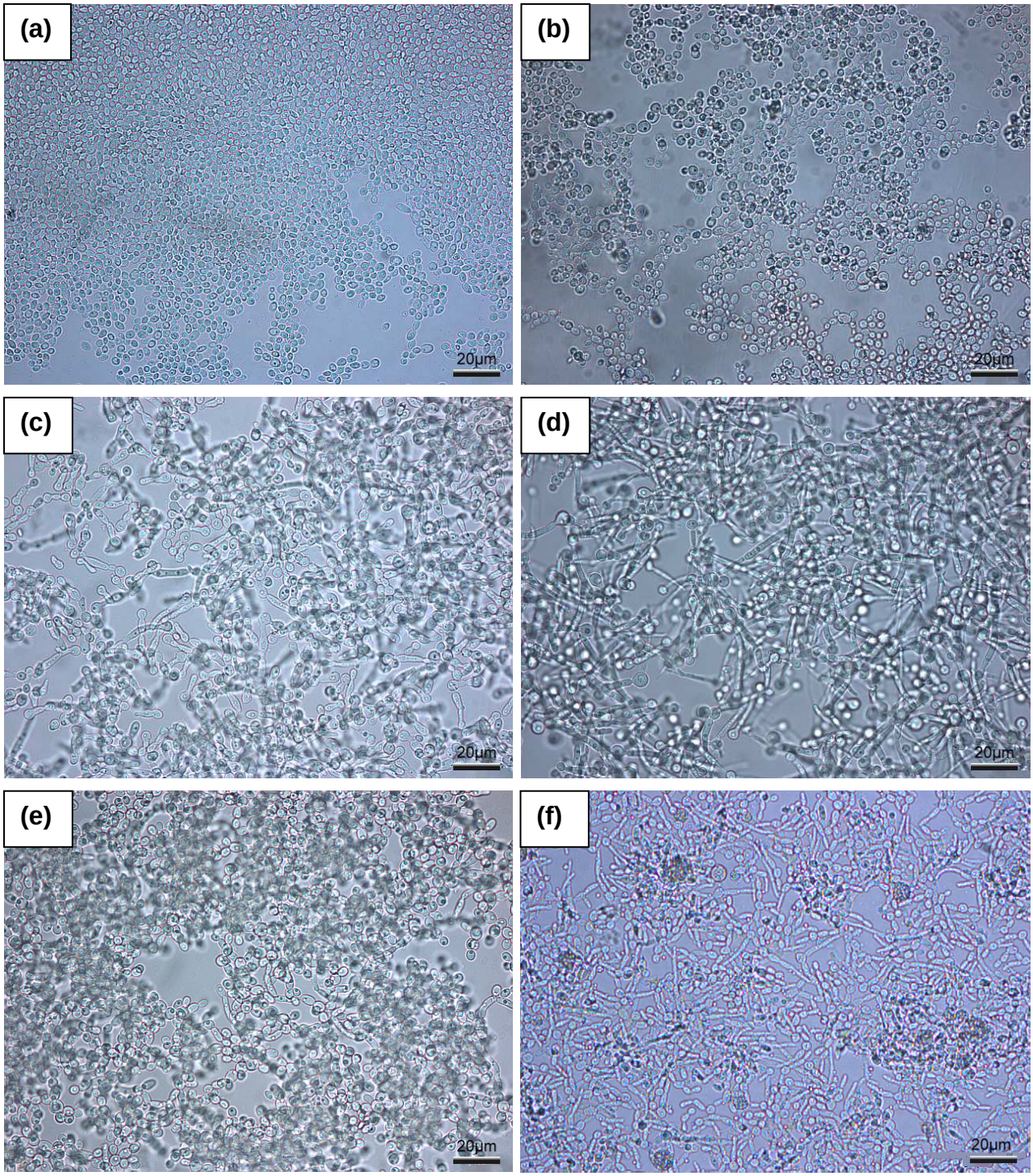


Fig. 3. Light micrographs of mature *Candida* biofilms grown for 48 h at 37 ℃ in chamber slides in the absence and presence of 1 mM arachidonic acid (20:4). (a) *C. albicans* CBS 562T absence of 20:4. (b) *C. albicans* CBS 562T presence of 20:4. (c) *C. albicans* NRRL Y-0477 absence of 20:4. (d) *C. albicans* NRRL Y-0477 presence of 20:4. (e) *C. dubliniensis* NRRL Y-17841T absence of 20:4. (f) *C. dubliniensis* NRRL Y-17841T presence of 20:4.

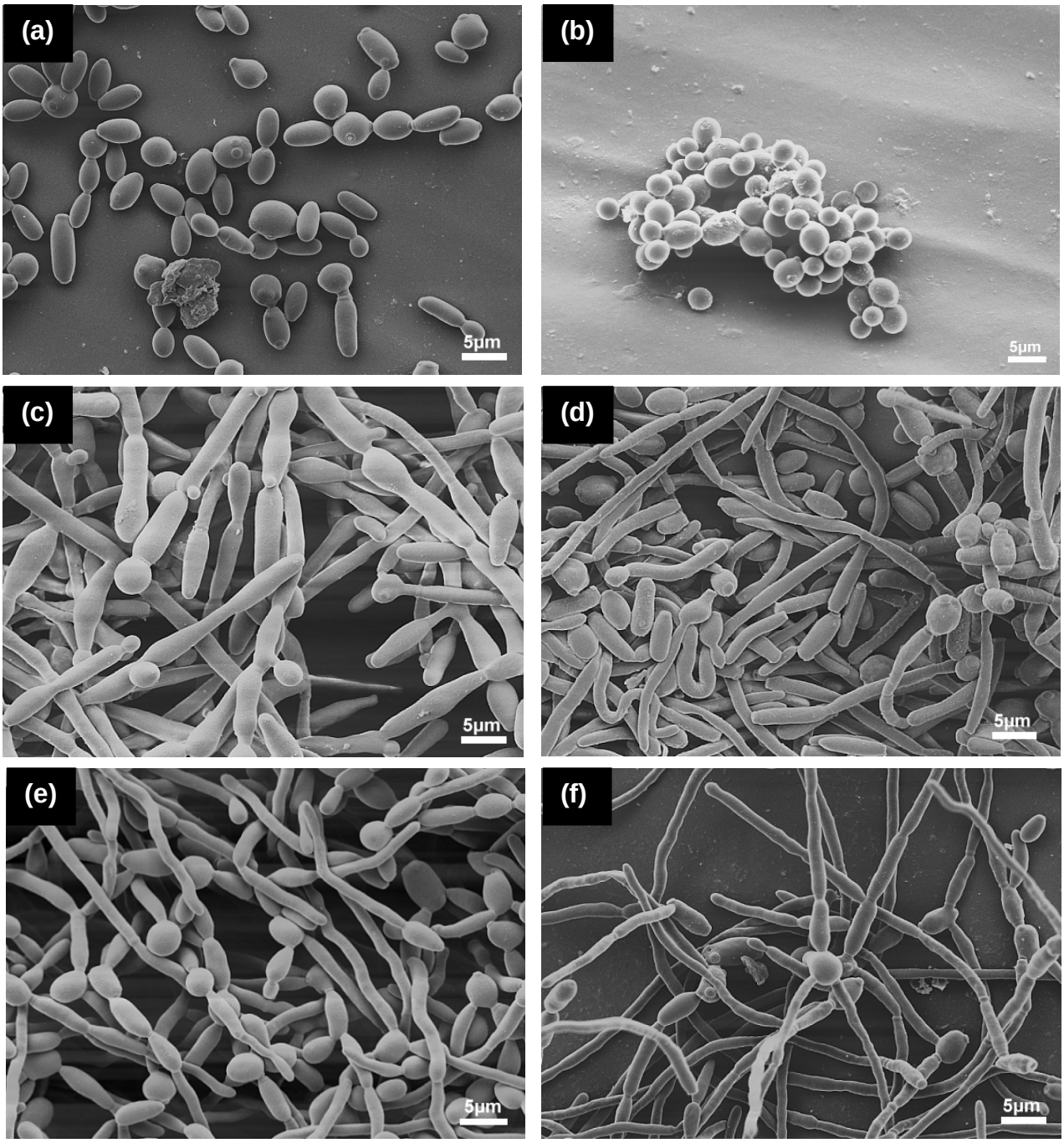


Fig. 4. Scanning electron micrographs of mature *Candida* biofilms grown for 48 h at 37 ℃ on silicone disks in chamber slides i n the absence and presence of 1 mM arachidonic acid (20:4). (a) *C. albicans* CBS 562T absence of 20:4. (b) *C. albicans* CBS 562T presence of 20:4. (c) *C. albicans* NRRL Y-0477 absence of 20:4. (d) *C. albicans* NRRL Y-0477 presence of 20:4. (e) *C. dubliniensis* NRRL Y-17841T absence of 20:4. (f) *C. dubliniensis* NRRL Y-17841T presence of 20:4.

b) Confocal laser scanning microscopy

The LIVE/DEAD® *Bac*Light™ Bacterial Viability kit was used to assess the viability of the cells in the mature biofilm. SYTO-9 is a nucleic acid stain that stains both the live and dead cells with a green fluorescence and PI is a red fluorescing nucleic acid dye that stains only the dead cells since it can only penetrate damaged cell membranes (Jin *et al*., 2005). These stains were used to evaluate viability of the *Candida* biofilms in the presence of 1 mM 20:4.

From the results (Fig. 5), it was again clear that the biofilms consist of yeast cells, budding yeast cells and/or filamentous forms. A greater part of the biofilms grown in the absence of 20:4 consists of live cells (green fluorescence) but they also contained some dead cells (red fluorescence). This might be due to oxygen and nutrient limitation as a result of the increasing biomass. The dead cells in the biofilm were found to be dispersed throughout the biofilm and not only in the middle layer as was found by Jin *et al*. (2005). The biofilms grown in the presence of 1 mM 20:4 also consisted out of yeast cells, hyphae and/or pseudohyphae and it is also clear that 1 mM 20:4 had no effect on the viability of these *Candida* strains.

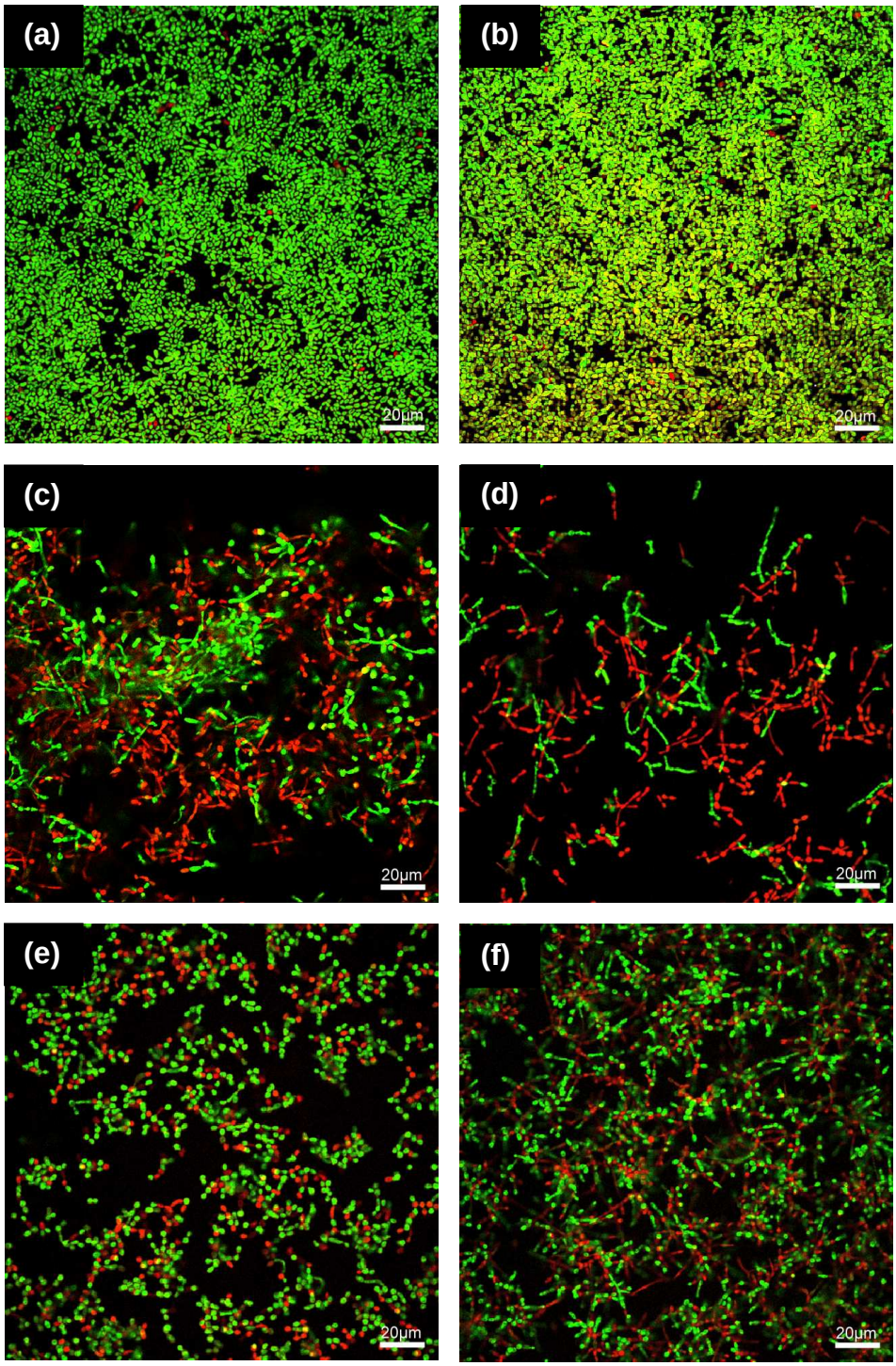


Fig. 5. Confocal laser scanning micrographs of mature *Candida* biofilms grown for 48 h at 37 ℃ in chamber slides in the absence and presence of 1 mM arachidonic acid (20:4). The biofilms were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit. (a) *C. albicans* CBS 562T absence of 20:4. (b) *C. albicans* CBS 562T presence of 20:4. (c) *C. albicans* NRRL Y-0477 absence of 20:4. (d) *C. albicans* NRRL Y-0477 presence of 20:4. (e) *C. dubliniensis* NRRL Y-17841T absence of 20:4. (f) *C. dubliniensis* NRRL Y-17841T presence of 20:4.

3.4.4) Effect of 20:4 on the metabolic activity of biofilms

The XTT assay is useful to examine biofilms, as it allows the study of the intact biofilm without interfering with the structure of the biofilm (Kuhn *et al*., 2003). As can be seen from Fig. 6, 20:4 had an inhibitory effect on mitochondrial activity during the early phase of biofilm formation by the *Candida* strains. For *C. albicans* CBS 562T (Fig. 6a) this inhibition was significant only during the initial stages of biofilm formation (0-11 h), but for *C. albicans* NRRL Y-0477 (Fig. 3b) the entire early phase of biofilm formation (0-24 h) was inhibited. The same was observed for *C. dubliniensis* NRRL Y-17841T (Fig. 6c), but with an inhibition continuing into the intermediate phase (12-30 h). However, in all cases the inhibitory effect was overcome and no difference could be seen between mature biofilms (48 h).

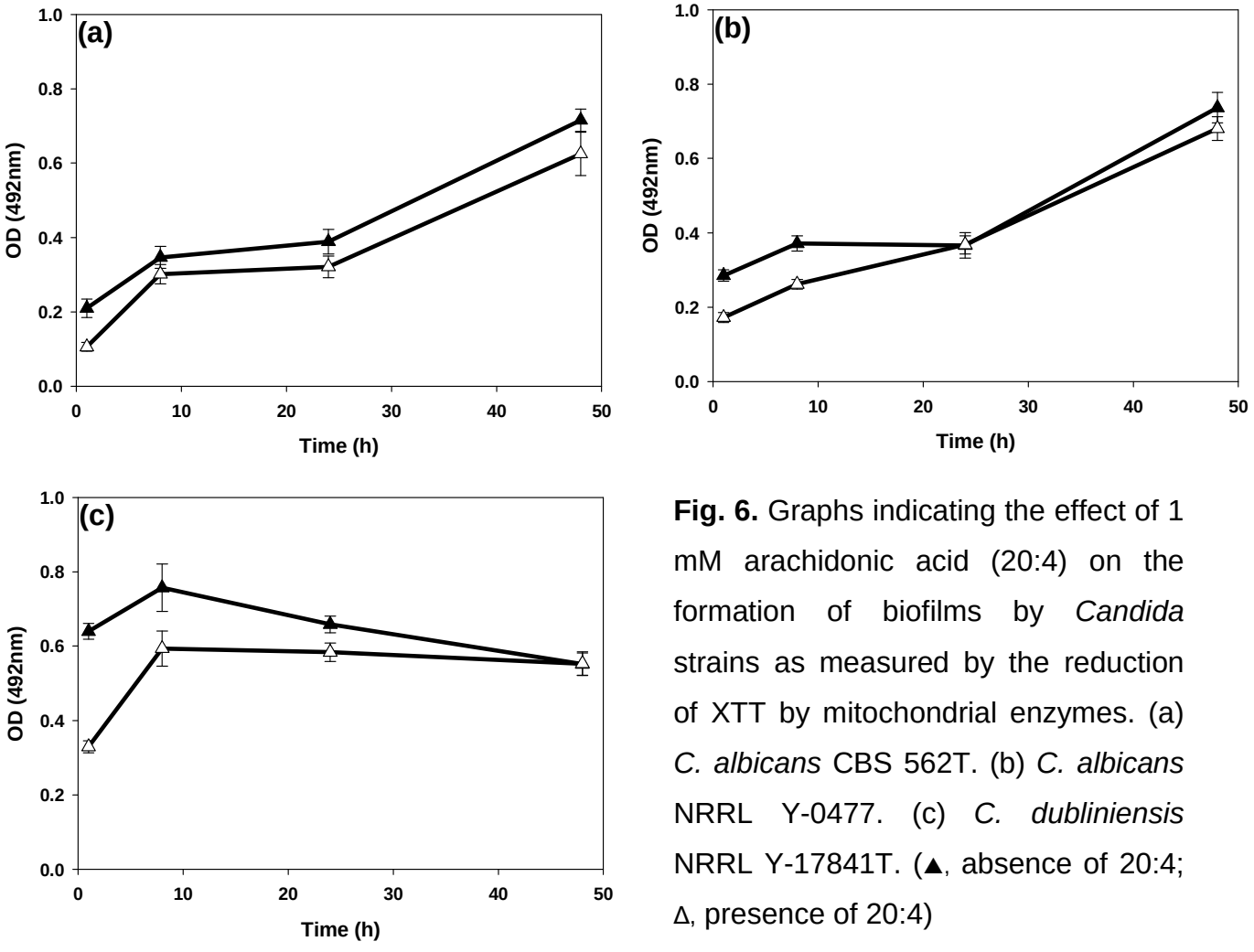


Fig. 6. Graphs indicating the effect of 1 mM arachidonic acid (20:4) on the formation of biofilms by *Candida* strains as measured by the reduction of XTT by mitochondrial enzymes. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-0477. (c) *C. dubliniensis* NRRL Y-17841T. (▲, absence of 20:4; Δ, presence of 20:4)

3.5) Conclusions

It was found that 1 mM 20:4 had little to no effect on the planktonic growth of the selected *C. albicans* and *C. dubliniensis* strains. It can also be concluded that although there were variations in the metabolic activity (measured by XTT reduction) between the strains, 1 mM 20:4 does not have any effect on the metabolic activity of the planktonic cells. However, 1 mM 20:4 had an inhibiting effect on the metabolic activity of biofilms at early and intermediate phases. However, during the maturation phase the inhibitory effect was overcome by all three strains and no difference could be seen between mature biofilms grown in absence and presence of 20:4. This was confirmed by the observation that 1 mM 20:4 does not influence the viability of cells in the biofilms. In addition, 1 mM 20:4 had no effect on the morphology of the cells in mature biofilms, indicating that it does not influence morphogenesis.

The study of lipids of pathogenic fungi has gained more interest recently, due to the possible involvement of lipids in pathogenesis and morphogenesis (Deva *et al*., 2000; Murayama *et al*., 2006). Since 20:4 is implicated in virulence mechanisms of *Candida* it would be important to study the metabolism of 20:4 in these *Candida* strains.

3.6) References

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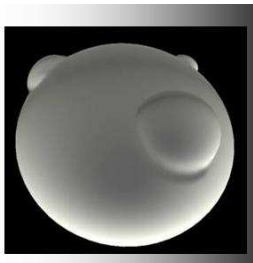
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CHAPTER 4

Arachidonic acid metabolism of selected *Candida* strains

4.1) Abstract

The uptake of long-chain fatty acids by yeasts is necessary for utilisation as metabolic fuels, cellular building blocks and the production of signalling molecules. This uptake is very complex and not fully understood, but certain mechanisms are proposed. Although arachidonic acid (20:4), a long-chain polyunsaturated fatty acid (PUFA), plays important roles during *C. albicans* infections, there are no definitive studies regarding the uptake and cellular metabolism of this compound by this pathogenic yeast. Therefore the aim of this study was to evaluate the uptake and incorporation of 20:4 by planktonic cells and biofilms of *C. albicans* and *C. dubliniensis*, as well as the identification of eicosanoids produced from 20:4 by biofilms of the selected strains. In order to do this, residual and cellular lipids were extracted from planktonic cells and biofilms during growth of these strains in the presence and absence of 20:4. The lipids were subjected to gas chromatography (GC) as well as gas chromatography-mass spectrometry (GC-MS). Strain specific variation in 20:4 uptake and incorporation into different lipid fractions of planktonic cells and biofilms were found. In addition it was found that 20:4 can be used by the biofilms of these strains to produce 3-hydroxy fatty acids.

4.2) Introduction

Fatty acids play important roles in cellular activity of fungi, including phospholipid synthesis, maintenance of membrane permeability, cell signalling, protein export and modification, and as a source of metabolic energy through β-oxidation (Black & DiRusso, 2003; Hamilton, 1998; Zou *et al*., 2003). These fatty acids may be synthesised *de novo* inside the cells, hydrolysed from triglycerides, or membrane lipids, or transported into the cells from the extracellular environment (Schaffer, 2002). It is currently accepted that transport of these hydrophobic fatty acids (with ionisable carboxyl groups) across the complex biological membranes occur either through passive diffusion, where no proteins are involved, or through more complex processes which involves membrane proteins and enzymes (Schaffer, 2002; Zou *et al*., 2003).

It was found that both these mechanisms might play a role, but under different conditions (Schaffer, 2002). The non-protein-mediated transport of fatty acids

proceeds in three steps i.e. adsorption, transmembrane movement and finally, desorption (Hamilton, 1998). During the first step, unbound fatty acids, in the extracellular environment, have to adsorb to the outer phospholipid layer of the cell membrane (Kamp & Hamilton, 1992). Following this, the fatty acid passively diffuses (flip-flop) over to the inner phospholipid layer of the membrane and is positioned so that the carboxyl group faces towards the cytoplasm (Civelek *et al*., 1996; Hamilton *et al*., 1994). This is known as transmembrane movement. It is also known that ionised fatty acids do not flip as rapidly as the un-ionised forms (Kamp & Hamilton, 1992). The movement of fatty acids is from a higher concentration (extracellular environment) to a lower concentration (intracellular environment), which is known as passive diffusion (Civelek *et al*., 1996; Hamilton *et al*., 1994). The last step in fatty acid uptake is desorption of the fatty acid from the phospholipid layer of the cell (Hamilton, 1998). Inside the cell, the un-ionised fatty acids release hydrogen ions from the carboxyl groups (Civelek *et al*., 1996; Hamilton *et al*., 1994). This release decreases the intracellular pH and creates a concentration gradient that further drives the system, providing an unsaturable transport system (Schaffer, 2002). The uptake of fatty acids by these three steps happens within seconds and usually dominates when the concentration of the available fatty acids is high. In contrast to this, transport of low concentrations of fatty acids involves protein-mediated uptake and leads to saturable transport.

The proteins which are involved in the transport and activation of fatty acids in eukaryotic cells, have been identified as fatty acid translocase (FAT), fatty acid transport protein (FATP) and fatty acyl-CoA synthetase (FACS) (Abumrad *et al*., 1993; 1999). This mechanism was named vectorial acylation and is based on the principle that the un-ionised fatty acid, that is collected by FAT and then bound to the outer phospholipid membrane, flips to the inner phospholipid membrane by the action of FATP and desorbs from it while it is activated by FACS (Black & Dirusso, 2007; Schaffer, 2002). The result is that fatty acids are released inside the cell as fatty acyl CoA esters. This mechanism is important in the transport of long-chain fatty acids, such as 20:4. After transport into the cell, the fatty acids can be incorporated into the different lipid fractions and used for protein acylation, as carbon and energy source through β-oxidation as well as for eicosanoid production (Jia *et al*., 2007).

Although it is known that *C. albicans* is able to utilise 20:4 as a carbon source - specifically via β-oxidation and the glyoxylate shunt of the citrate cycle rather than being oxidised by the respiratory chain or the citrate cycle - for the production of energy (Deva *et al*., 2000), and as a precursor for eicosanoid production (Goyal & Khuller, 1994; Noverr *et al*., 2001), little attention has been paid to the uptake and incorporation of this fatty acid by this yeast and no data are available on 20:4 metabolism by *C. dubliniensis*. Therefore, the aim of this chapter became to evaluate the uptake and utilisation of exogenous 20:4 by planktonic cells and biofilms as well as the production of eicosanoids from 20:4 by biofilms of the selected *Candida* strains.

4.3) Materials and methods

4.3.1) Strains used

The following strains were used in this study: *Candida albicans* CBS 562T, *C. albicans* NRRL Y-0477, *C. dubliniensis* NRRL Y-17841T. All strains were maintained on yeast malt extract (YM) agar [10 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone, 16 g agar, 1000 mL distilled water (dH_20)] at room temperature.

4.3.2) Uptake of 20:4 by planktonic cells

Candida strains, from 24 h old cultures on YM agar plates, were aseptically inoculated into 200 mL sterile yeast nitrogen base (YNB) broth [6.7 g YNB, 1000 mL dH_20] containing 10 $g.L^{-1}$ glucose and incubated aerobically in 500 ml conical flasks at 37 ℃ for 12 h. The cells were harvested by centrifugation (10 min at 4000 *g*), counted and diluted to 1 x 10^6 $cells.mL^{-1}$ in 100 mL YNB broth with 1 mM 20:4 (30.5 $g.L^{-1}$) (Sigma-Aldrich, USA) (in ethanol) and incubated aerobically at 37 ℃. Samples were taken at 0 h, 6 h and 12 h. A control experiment containing 10 $g.L^{-1}$ glucose as sole carbon source was also performed. Each sample was centrifuged (10 min at 4000 *g*) and the supernatant decanted. The pH of the supernatant was adjusted to below 4.0 with 3 % (v/v) formic acid and two volumes ethyl acetate (Lasec, SA) were added. The organic phase was removed, lauric acid (12:0) (Fluka, Switzerland) was added as internal standard and the organic phase was left to

evaporate overnight. After evaporation, the lipids were transferred to preweighed vials and the fatty acids methylated with trimethyl sulphonium hydroxide (TMSOH) (Butte, 1983). The fatty acid methyl esters were analysed on a Shimadzu GC-2010 gas chromatograph (GC) with a flame ionization detector. A SGE-BPX-70 column of 60 m in length with an inner diameter of 0.32 mm with nitrogen as the carrier gas at a flow rate of 0.5 $mL.min^{-1}$ was used. One microlitre of the sample was injected into the GC. The fatty acids were determined by reference to authentic standards and the concentration of 20:4 was calculated.

4.3.3) Incorporation of 20:4 by planktonic cells

The cell pellets from the above procedure, obtained at every time interval, were washed twice with dH_2O and the wet mass of the cells determined. The cells were ground with a mortar and pestle and the neutral lipids (NLs) were extracted with hexane (Lasec, SA) for 6 h. After filtration through lipid free filter paper, 12:0 was added as internal standard. The NLs were weighed and analysed by GC as described above.

The cells were removed from the filter paper and, in order to extract the glycolipids (GLs) and the phospholipids (PLs), chloroform/methanol (2 : 1, v/v) (Lasec, SA) was added and left overnight. The extracted lipids were washed and the solvent evaporated under vacuum in a rotary evaporator (Folch *et al*., 1957). The lipids were transferred to preweighed glass vials. The GL and PL fractions were separated by column chromatography with heat activated (110 ℃) silicic acid (100-200 mesh, Sigma, USA) as the stationary phase (Kock & Ratledge, 1993). The GLs were eluted with 100 ml acetone (Lasec, SA) and the PLs with 100 ml methanol. Lauric acid was added as internal standard to the GL fraction before evaporation under vacuum in a rotary evaporator. The GL fraction was methylated and analysed on the GC as described above.

The PLs were separated into different fractions [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS)] by silica gel thin layer chromatography (MERCK, Germany) at 4 ℃ (Kock & Ratledge, 1993). The mobile phase was chloroform/methanol/acetic acid/water (65 : 43 : 3 : 1, by vol.). The PL fractions were visualised with iodine vapour and each band was scraped off and extracted with

chloroform/methanol (2 : 1, v/v) for 4-6 h. Each fraction was then filtered through lipid free filter paper into preweighed vials and evaporated before GC analysis as described above.

The unsaturation indices of the NLs, GLs and PLs were calculated using the following formula: $\Delta mole^{-1}$ = [1 x (% monoene)]/100 + [2 x (% diene)]/100 + [3 x (% triene)]/100 (Mishra & Prasad, 1989).

4.3.4) Uptake and incorporation of 20:4 by biofilms

The strains, from 24 h old cultures on YM agar plates, were aseptically inoculated into 20 mL sterile YNB broth containing 10 $g.L^{-1}$ glucose in 50 mL sterile centrifuge tubes and incubated at 30 ℃ for 48 h. The cells were washed twice with phosphate buffered saline (PBS) (OXOID, UK) and resuspended into filter sterilised RPMI-1640 medium (Sigma-Aldrich, USA). The cells were counted and diluted to 1 x 10^6 $cells.mL^{-1}$ in 600 mL filter sterilised RPMI-1640 medium containing a final concentration of 1 mM 20:4. Flasks with appropriate controls were also included. These suspensions were poured into polystyrene Petri dishes and incubated at 37 ℃ for up to 48 h, to allow biofilm formation (Ramage *et al*., 2001). Samples were taken at 0 h, 1 h, 8 h, 24 h and 48 h. The samples were analysed as for the planktonic cells, to determine the uptake and incorporation of 20:4, except that the biofilms were scraped from the plates with cell scrapers (Costar®, Corning Incorporated, NY) and added into preweiged eppendorf tubes before centrifugation and extraction. The unsaturation indices of the different lipid fractions were determined as described above.

4.3.5) Eicosanoid production

Biofilms were grown as above in polystyrene Petri dishes in the absence and presence of 1 mM 20:4. The cells were scraped off and added, with the supernatant, into clean conical flasks. The pH was adjusted to below 4.0 with 3 % (v/v) formic acid and two volumes ethyl acetate were added. The organic phase was removed and left to evaporate overnight before the lipids were methylated with TMSOH and silylated with bis(trimethylsilyl)trifluoroacetamide (MERCK, Germany) and pyridine (MERCK, Germany) at room temperature (Venter *et al*., 1997). The samples were dried under nitrogen gas and

dissolved in chloroform/hexane (4 : 1, v/v) for gas chromatography-mass spectrometry (GC-MS) analyses. The samples were injected into a Finnigan Trace GC Ultra gas chromatograph (Thermo Electron Corporation, San Jose, Calif., USA) with a HP5 (60 m x 0.32 mm diameter) fused silica capillary column (0.1 µm coating thickness) coupled to a Finnigan Trace DSQ mass-spectrometer (Thermo Electron Corporation). The carrier gas was helium at 1.0 $mL.min^{-1}$. The initial oven temperature of 110 ℃ was mainta ined for 2 min and then increased to a final temperature of 280 ℃ at a rate of 5 $℃.min^{-1}$. One microlitre of the sample was injected into the GC-MS at a split ratio of 1:50 at an inlet temperature of 230 ℃ (Venter *et al*., 1997).

4.3.6) Statistical analysis

All experiments were performed at least in triplicate. The *t*-test was performed to determine if the data sets obtained were significantly different from each other with a P-value equal to or less than 0.05.

4.4) Results and discussion

4.4.1) Uptake of 20:4 by planktonic cells

From the results it seems that 20:4 uptake by *C. albicans* strains occurs via an unsaturable transport mechanism since 20:4 is taken up over the whole 12 h period (Fig. 1). There were, however differences in the rate of uptake between the two *C. albicans* strains after 6 h, as *C. albicans* CBS 562T (Fig. 1) took up more 20:4 (*ca* 50 %) compared to *C. albicans* NRRL Y-0477 (*ca* 20 %). However, after 12 h all three strains took up similar amounts of 20:4. In contrast to these findings, it was found that *C. dubliniensis* NRRL Y-17841T probably has a saturable transport mechanism, allowing for the rapid uptake (*ca* 70%) of 20:4 only during the first 6 h of growth. This transport mechanism observed for *C. dubliniensis* NRRL Y-17841T is unexpected, since a relatively high concentration 20:4 was available. It must be noted that these observed differences are not due to differences in growth. In Chapter 3 (Fig. 1) it was indicated that these strains grew to the same extent in the presence of 20:4.

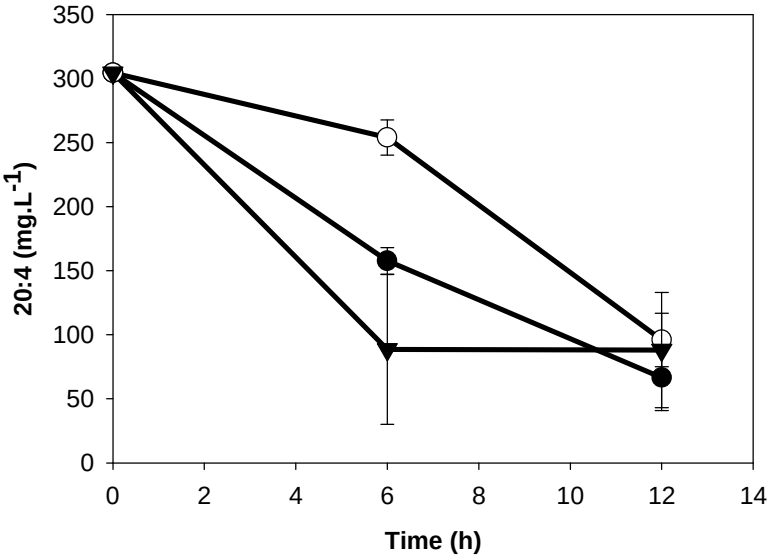


Fig. 1. Graphs indicating the uptake of arachidonic acid (20:4) by planktonic *Candida* strains after incubation at 37 ℃ for up to 12 h in the presence of 1 mM 20:4. (●, *C. albicans* CBS 562T; o, *C. albicans* NRRL Y-0477; ▼, *C. dubliniensis* NRRL Y-17841T)

4.4.2) Metabolic fate of 20:4 in planktonic cells

When fatty acids are taken up by an organism they can be used for energy production through β-oxidation in the mitochondria or peroxisomes, and for the synthesis of other fatty acids or lipids and eicosanoids (Jia *et al*., 2007; Kamp & Hamilton, 1992; Schaffer, 2002; Tehlivets *et al*., 2007). The fate of 20:4 in planktonic cells was determined and, as can be seen in Table 1 only a fraction of the 20:4 taken up, is incorporated into the biomass as part of the cellular lipids (i.e. NL, GL and PL) of the three strains. *Candida dubliniensis* NRRL Y-17841T incorporates more 20:4 into the cellular lipids than the *C. albicans* strains. Interestingly, the level of 20:4 in the cellular lipids of *C. albicans* strains remained constant but increased for *C. dubliniensis* NRRL Y-17841T, as cells enter the stationary phase (12 h). Although there are differences in the amount of 20:4 available for cellular metabolism (other than incorporation into cellular lipids), these levels remain constant for *C. dubliniensis* NRRL Y-17841T during the growth period but increased for the two *C. albicans* strains over the growth period.

Table 1: Arachidonic acid turnover by planktonic *Candida* strains.

Strains	Time (h)	20:4 incorporated into lipids (mg)*	20:4 available for metabolism (mg)†
C. albicans CBS 562T	**6**	1.1 ± 0.5	13.7 ± 1.6
	12	1.4 ± 0.8	22.4 ± 3.0
C. albicans NRRL Y-0477	**6**	0.6 ± 0.2	4.7 ± 1.6
	12	0.6 ± 0.3	20.2 ± 2.8
C. dubliniensis NRRL Y-17841T	**6**	2.9 ± 0.2	18.7 ± 5.6
	12	5.6 ± 2.1	16.0 ± 2.5

*20:4 incorporated into the cellular lipids of planktonic *Candida* strains.
†20:4 available for cellular metabolism (e.g. β-oxidation, eicosanoid production etc.), i.e. 20:4 not incorporated into lipids of planktonic *Candida* strains.

The 20:4 incorporated into the cellular lipids were found to be incorporated into the NLs and GLs (Fig. 2, 3, 4), but not into the PLs of the planktonic *Candida* strains. In *C. albicans* CBS 562T (Fig. 2) and *C. albicans* NRRL Y-0477 (Fig. 3) the concentration 20:4 increases in the NLs and the GLs during the first 6 h of growth and thereafter no significant increase was found up to 12 h. In *C. dubliniensis* NRRL Y-17841T (Fig. 4), there is a significant increase in the concentration 20:4 in the NLs during first 6 h of growth, followed by a further slight increase. In the GLs of this strain there is a significant increase over the whole growth period. These results are similar to literature. Deva *et al*. (2000) also found 20:4 in the GLs and monoacylglycerols (NLs) but not in the PL fraction of planktonic *C. albicans* after growth in the presence of 10 µM 20:4.

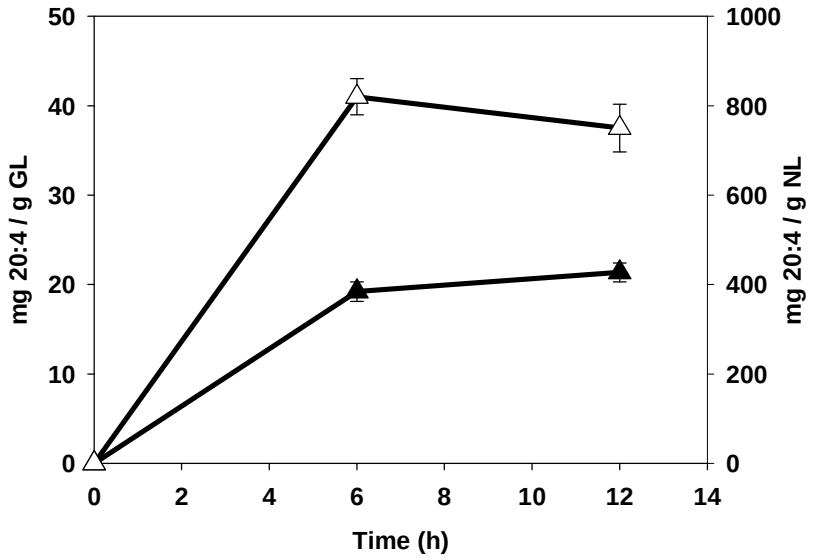


Fig. 2. Graphs indicating the incorporation of arachidonic acid (20:4) into the neutral lipids (NL) and the glycolipids (GL) of planktonic *Candida albicans* CBS 562T after growth in the presence of 1 mM 20:4 at 37 ℃ for up to12 h. [▲, mg 20:4 / g GL; Δ, mg 20:4 / g NL]

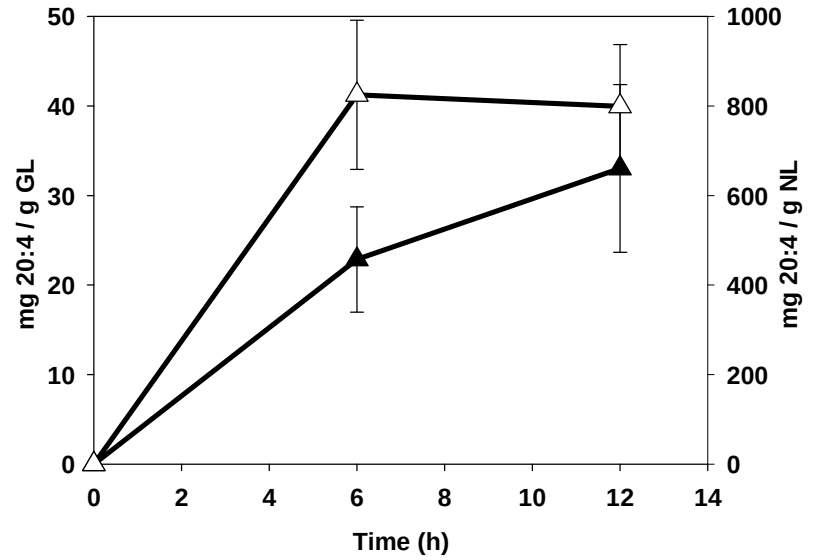


Fig. 3. Graphs indicating the incorporation of arachidonic acid (20:4) into the neutral lipids (NL) and the glycolipids (GL) of planktonic *Candida albicans* NRRL Y-0477 after growth in the presence of 1 mM 20:4 at 37 ℃ for up to12 h. [▲, mg 20:4 / g GL; Δ, mg 20:4 / g NL]

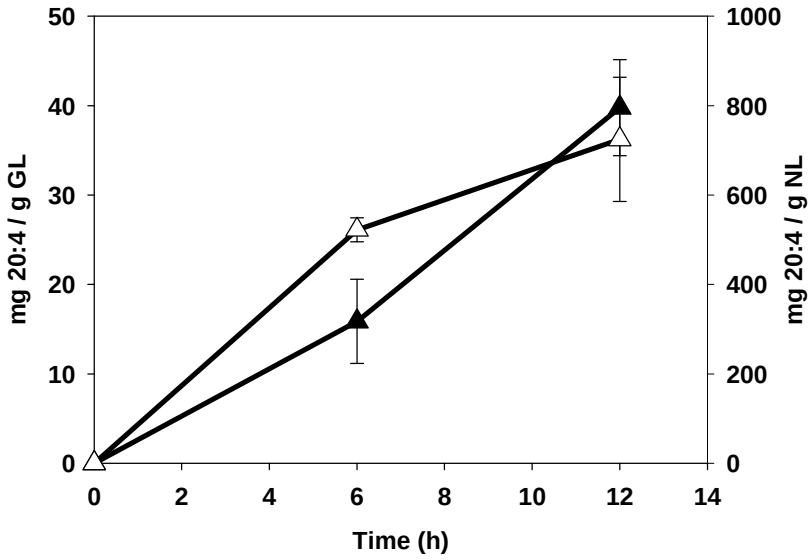


Fig. 4. Graphs indicating the incorporation of arachidonic acid (20:4) into the neutral lipids (NL) and the glycolipids (GL) of planktonic *Candida dubliniensis* NRRL Y-17841T after growth in the presence of 1 mM 20:4 at 37 ℃ for up to 12 h. [▲, mg 20:4 / g GL; Δ, mg 20:4 / g NL]

When the effect of 20:4 on the unsaturation indices of the cellular lipid fractions was examined (Table 2) it became clear that 20:4 increased the unsaturation of the NLs of all three strains and the GLs of only *C. albicans* NRRL Y-0477 and *C. dublini*ensis NRRL Y-17841T, but did not significantly affect the unsaturation index of the PLs of any of the *Candida* strains. This indicates that 20:4 had no effect on the unsaturation of the planktonic cell membranes. It is postulated that these *Candida* strains might have a mechanism for regulating the unsaturation in the membrane phospholipids even in the presence of exogenous PUFAs.

Table 2: The unsaturation index ($\Delta mole^{-1}$)* of the neutral (NL), glyco- (GL) and phospholipids (PL) of planktonic *Candida* strains grown in the absence (control) and presence of arachidonic acid (20:4).

		***C. albicans* CBS 562T**			***C. albicans* NRRL Y-0477**			***C. dubliniensis* NRRL Y-17841T**		
	Time (h)	**NL**	**GL**	**PL**	**NL**	**GL**	**PL**	**NL**	**GL**	**PL**
Control	**0**	0.6 ± 0.4	0.7 ± 0.2	0.3 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.5 ± 0.1
	6	0.3 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	0.7 ± 0.5	0.5 ± 0.2
	12	0.5 ± 0.1	0.7 ± 0.2	0.6 ± 0.2	0.2 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.1	0.5 ± 0.0
20:4	**0**	0.6 ± 0.4	0.7 ± 0.2	0.2 ± 0.0	0.4 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.4 ± 0.1
	6	3.4 ± 0.5	1.1 ± 0.4	0.3 ± 0.1	3.6 ± 0.1	2.0 ± 0.1	0.2 ± 0.1	1.7 ± 0.9	1.2 ± 0.0	0.6 ± 0.1
	12	3.3 ± 0.5	1.3 ± 0.6	0.5 ± 0.2	2.9 ± 0.2	2.0 ± 0.3	0.4 ± 0.1	2.1 ± 1.5	1.9 ± 0.1	0.4 ± 0.0

*$\Delta mole^{-1}$ = [1 x (% monoene)]/100 + [2 x (% diene)]/100 + [3 x (% triene)]/100

4.4.3) Uptake of 20:4 by biofilms

The results indicate that *C. albicans* CBS 562T biofilm probably has an unsaturable 20:4 uptake (Fig. 5), similar to the planktonic cells (Fig. 1). This is confirmed by the rapid uptake of 20:4 during the first hour of growth, i.e. during the adhesion phase, which might indicate passive diffusion (flip-flop) of 20:4 across the membranes. Interestingly, it was found that *C. albicans* NRRL Y-0477 biofilm probably has a saturable uptake (Fig. 5), which differs from the mechanism in planktonic cells (Fig. 1). This is confirmed by the lack of significant 20:4 uptake during the first eight hours of growth. This uptake mechanism is again unexpected under these conditions, since a relatively high 20:4 concentration was available. Since it is known that protein expression of *C. albicans* differs between planktonic and biofilm cells (Seneviratne *et al.*, 2008) it may be speculated that certain fatty acid transport proteins of this strain are only expressed when the yeast grows as biofilms.

For *C. dubliniensis* NRRL Y-17841T biofilm (Fig. 5), the same saturable uptake was observed as for the planktonic cells (Fig. 1). This was also indicated by a rapid uptake of 20:4 during the first hour of growth. However, after this there is a gradual and eventually saturable uptake of 20:4, which might indicate the presence of membrane proteins responsible for the transport of fatty acids as the concentration 20:4 decreases. This may be an example of the presence of both mechanisms in one organism, as indicated by Schaffer (2002). When interpreting this data it must, however, be noted that the differences in the levels of 20:4 uptake between the different strains may also be attributed to differences in the biomass produced by the different strains. In Chapter 3 (Fig. 5) it is clear that *C. albicans* CBS 562T and *C. dubliniensis* NRRL Y-17841T produce mature biofilms with higher cell numbers than *C. albicans* NRRL Y-0477, possibly explaining the lower level of 20:4 uptake by *C. albicans* NRRL Y-0477.

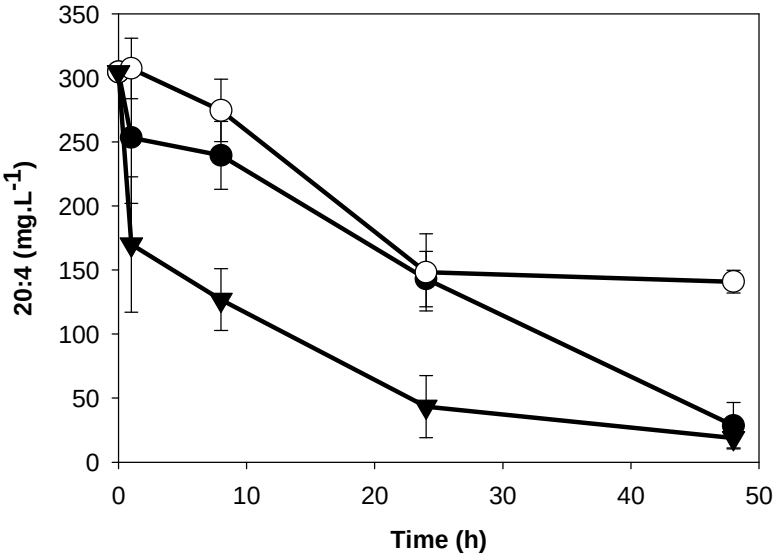


Fig. 5. Graphs indicating the uptake of arachidonic acid (20:4) by *Candida* biofilms after incubation at 37 ℃ for up to 48 h in the presence of 1 mM 20:4. (●, *C. albicans* CBS 562T; o, *C. albicans* NRRL Y-0477; ▼, *C. dubliniensis* NRRL Y-17841T)

4.4.4) Metabolic fate of 20:4 in biofilms

As in the case of the planktonic cells, the 20:4 taken up by these biofilms are incorporated into the biomass as part of the cellular lipids (i.e. NL, GL and PL) and used in cellular metabolism (Table 3). Similar to the planktonic cells, more 20:4 was available for cellular metabolism of biofilms of all the strains than was incorporated into the cellular lipids. Interestingly, even though more 20:4 was taken up by *C. dubliniensis* NRRL Y-17841T during the first hour of incubation, the levels of 20:4 in the cellular lipids of all three strains were similar. This rapid uptake of 20:4 by *C. dubliniensis* NRRL Y-17841T therefore made more 20:4 available for cellular metabolism at this time. In fact, throughout the incubation period, more 20:4 is available for cellular metabolism in *C. dubliniensis* NRRL Y-17841T than in *C. albicans* strains, except after 48 h when both *C. dubliniensis* NRRL Y-17841T and *C. albicans* CBS 562T had the same amount of 20:4 available for cellular metabolism.

Table 3: Arachidonic acid (20:4) turnover by *Candida* biofilms.

Strains	Time (h)	20:4 Incorporated into lipids (mg)*	20:4 available for metabolism (mg)†
C. albicans CBS 562T	**1**	0.2 ± 0.1	4.9 ± 1.9
	8	0.4 ± 0.1	6.1 ± 3.9
	24	1.0 ± 0.5	15.1 ± 2.4
	48	0.9 ± 0.1	26.7 ± 1.8
C. albicans NRRL Y-0477	**1**	0.2 ± 0.1	0.2 ± 0.2
	8	1.0 ± 0.6	2.0 ± 0.8
	24	2.6 ± 1.1	13.0 ± 4.6
	48	2.2 ± 1.7	14.1 ± 2.2
C. dubliniensis NRRL Y-17841T	**1**	0.3 ± 0.2	13.1 ± 5.5
	8	0.7 ± 0.2	17.0 ± 2.5
	24	1.3 ± 0.3	24.8 ± 2.5
	48	1.2 ± 0.1	27.3 ± 0.7

*20:4 incorporated into the cellular lipids of the biofilms of these *Candida* strains.

†20:4 available for cellular metabolism (e.g. β-oxidation, eicosanoid production etc.), i.e. 20:4 not incorporated into lipids of the biofilms of these *Candida* strains.

Arachidonic acid could be detected in all three lipid fractions of biofilms of all three *Candida* strains within the first hour of growth. In *C. albicans* CBS 562T (Fig. 6) there was an increase in the concentration 20:4 in the NLs during the early and the intermediate phase of biofilm formation, but after this no significant increase was found. In *C. albicans* NRRL Y-0477 (Fig. 7) and *C. dubliniensis* NRRL Y-17841T (Fig. 8) there was only an increase in the concentration 20:4 in the NLs during the early phase and no increase in the intermediate and mature phase. The 20:4 concentration was found to increase in the GLs of all three strains during biofilm formation with the most 20:4 incorporated into the GLs of *C. albicans* NRRL Y-0477 and the least into the GLs of *C. albicans* CBS 562T.

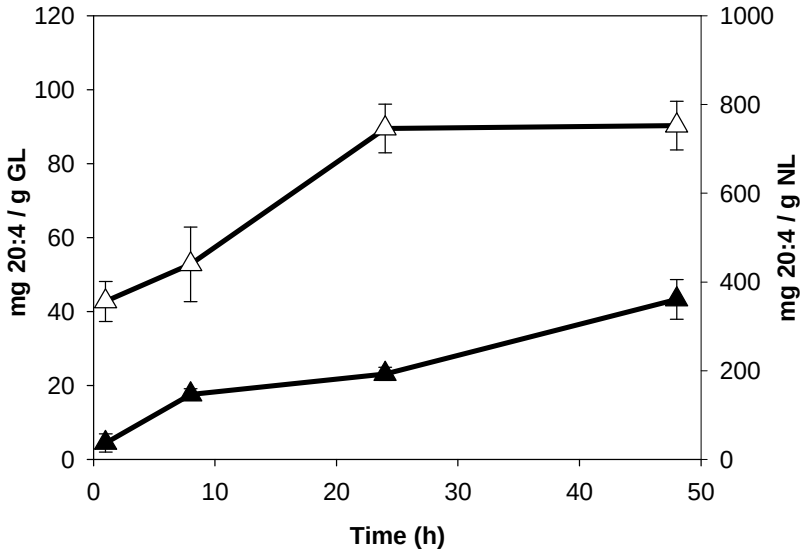


Fig. 6. Graphs indicating the incorporation of arachidonic acid (20:4) into the neutral lipids (NL) and the glycolipids (GL) of *Candida albicans* CBS 562T biofilm after growth in the presence of 1 mM 20:4 at 37 ℃ for up to 48 h. [▲, mg 20:4 / g GL; Δ, mg 20:4 / g NL]

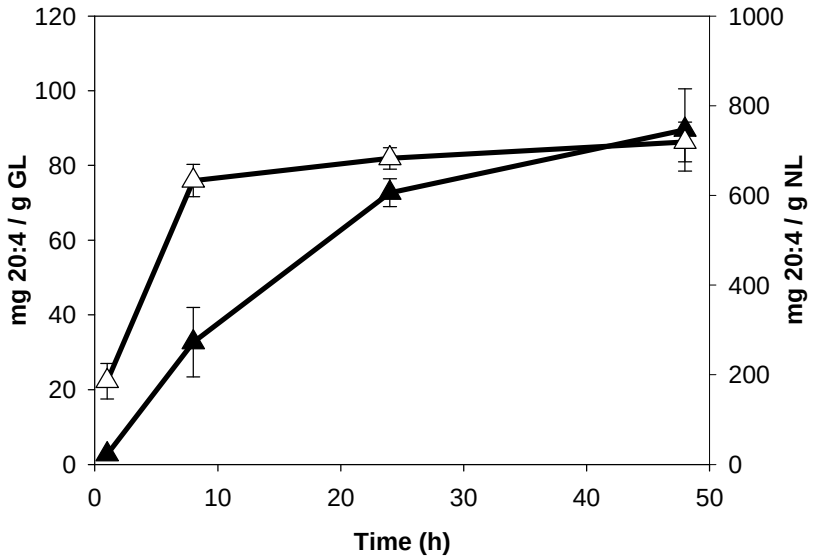


Fig. 7. Graphs indicating the incorporation of arachidonic acid (20:4) into the neutral lipids (NL) and the glycolipids (GL) of *Candida albicans* NRRL Y-0477 biofilm after growth in the presence of 1 mM 20:4 at 37 ℃ for up to 48 h. [▲, mg 20:4 / g GL; Δ, mg 20:4 / g NL]

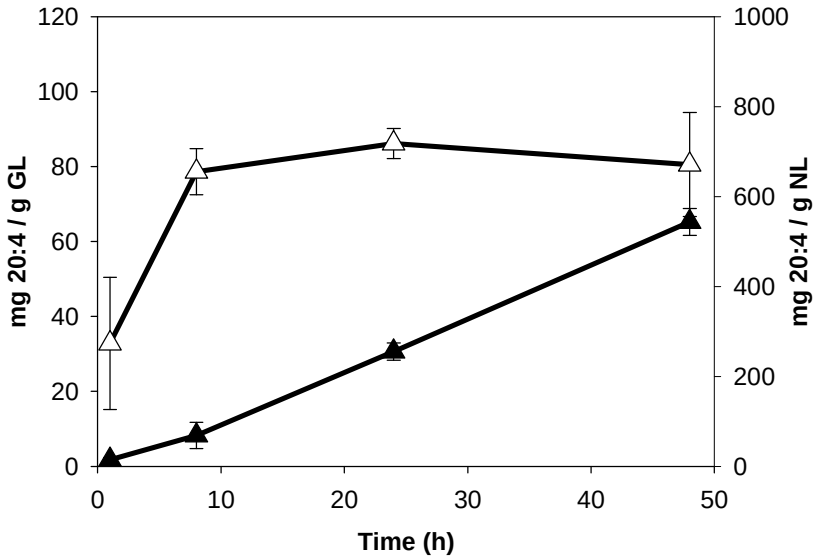


Fig. 8. Graphs indicating the incorporation of arachidonic acid (20:4) into the neutral lipids (NL) and the glycolipids (GL) of *Candida dubliniensis* NRRL Y-17841T biofilm after growth in the presence of 1 mM 20:4 at 37 ℃ for up to 48 h. [▲, mg 20:4 / g GL; Δ, mg 20:4 / g NL]

It was found that, in the presence of 20:4, there is a significant increase in the unsaturation index of the NLs and the GLs for all three strains (Table 4). The unsaturation index of the PLs of *C. albicans* CBS 562T was the only PL unsaturation index that had a significant increase even though 20:4 was incorporated into the PLs of all three strains. This suggests an efficient mechanism in the other two strains in order to maintain the optimum level of unsaturation in their cell membranes.

Table 4: The unsaturation index ($\Delta mole^{-1}$)* of the neutral (NL), glyco- (GL) and phospholipids (PL) of the *Candida* biofilms in the absence (control) and presence of arachidonic acid (20:4).

		***C. albicans* CBS 562T**			***C. albicans* NRRL Y-0477**			***C. dubliniensis* NRRL Y-17841T**		
	Time (h)	**NL**	**GL**	**PL**	**NL**	**GL**	**PL**	**NL**	**GL**	**PL**
Control	**1**	0.5 ± 0.2	0.6 ± 0.0	0.3 ± 0.0	0.5 ± 0.2	0.5 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.4 ± 0.0	0.2 ± 0.1
	8	0.4 ± 0.2	0.7 ± 0.0	0.2 ± 0.1	0.4 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.2 ± 0.0
	24	0.5 ± 0.4	0.6 ± 0.1	0.2 ± 0.0	0.5 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.2 ± 0.0
	48	0.5 ± 0.3	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
20:4	**1**	1.8 ± 0.2	1.0 ± 0.6	0.4 ± 0.1	1.2 ± 0.5	0.7 ± 0.2	0.4 ± 0.1	1.5 ± 0.6	0.7 ± 0.2	0.5 ± 0.1
	8	2.1 ± 0.3	2.0 ± 0.2	0.4 ± 0.0	2.7 ± 0.2	1.5 ± 0.1	0.4 ± 0.2	2.8 ± 0.4	0.9 ± 0.2	0.4 ± 0.1
	24	3.2 ± 0.1	2.1 ± 0.7	0.4 ± 0.1	3.1 ± 0.5	1.9 ± 0.5	0.4 ± 0.1	3.0 ± 0.2	1.5 ± 0.1	0.3 ± 0.1
	48	3.2 ± 0.3	1.7 ± 0.5	0.7 ± 0.0	3.0 ± 0.5	2.0 ± 0.5	0.4 ± 0.1	2.9 ± 0.4	1.9 ± 0.5	0.6 ± 0.1

*$\Delta mole^{-1}$ = [1 x (% monoene)]/100 + [2 x (% diene)]/100 + [3 x (% triene)]/100

4.4.5) Effect of 20:4 on the phospholipids of biofilms

Different PL fractions are synthesised by different biosynthetic pathways (Fig. 9) (Daum & Paltauf, 1990; de Kroon, 2007). In short, the enzymes responsible for PI and PS production from cytidine diphosphate-diacylglycerol (CDP-DAG) are phosphatidylinositol synthase and phosphatidylserine synthase respectively. Phosphatidylethanolamine is formed by the decarboxylation of PS by a decarboxylase and PC from three stepwise methylations of PE by methyltransferases. Phosphatidylcholine and PE might also be produced by a second pathway, the Kennedy pathway, when the corresponding precursors are supplied. Both these mechanisms have been observed in *C. albicans* (Klig *et al*., 1990).

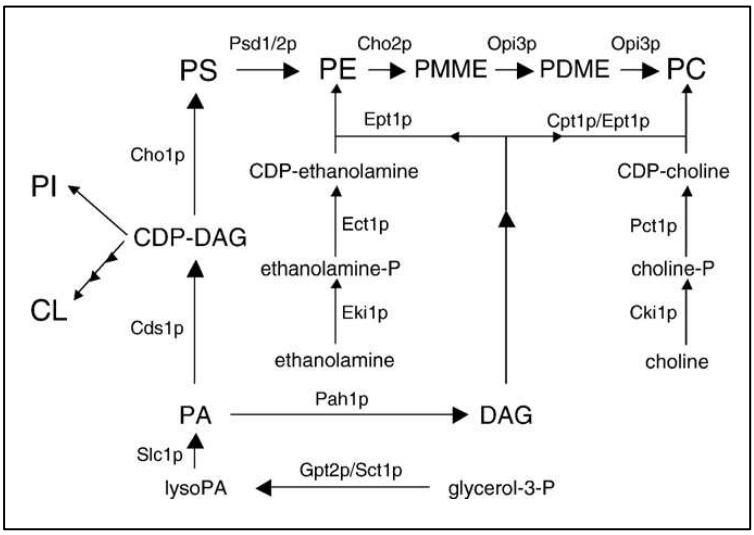


Fig. 9. The major phospholipid biosynthetic pathways in yeast indicating the enzymes responsible. (DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMME, phosphatidyl-monomethylethanolamine; PS, phosphatidylserine) (de Kroon, 2007).

It is known that PC and PE are the major phospholipid fractions in eukaryotic organisms (Abdi & Drucker, 1996; de Kroon, 2007; Deng *et al*., 2007). The results obtained in this study, indicated that the PC and PE content in *C. albicans* CBS 562T are between 20-50 % (w/w) and in *C. albicans* NRRL Y-0477 and *C. dubliniensis* NRRL Y-17841T between 20-30 % (w/w) of the total

PLs during biofilm formation. The PC and PE fractions are known to play important structural roles in the cell membranes and in the maintenance of membrane homeostasis (de Kroon, 2007). Membrane homeostasis can be maintained through the remodelling of the phospholipid fractions through deacylation - reacylation cycles (Pérez *et al*., 2006), where acyl chains are exchanged by phospholipase and acyltransferase activities in the Lands cycle (Lands, 2000) (Fig. 10). Different fatty acyl chains of PLs affect membrane fluidity by allowing different packaging (Lands, 2000) and is important in maintaining the correct fluidity of the cell membrane under different conditions, i.e. growth at different temperatures and the presence of different fatty acids (e.g. 20:4). It is believed that 20:4 is presented into PLs by this pathway (Yamashita *et al*., 1997).

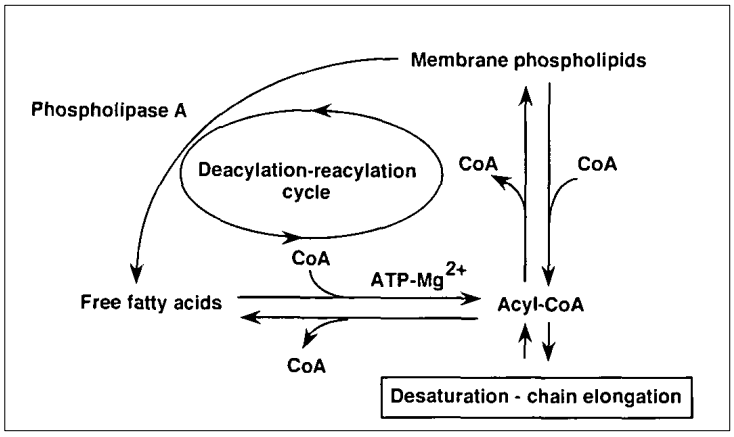


Fig. 10. The Lands cycle indicating the deacylation – reacylation steps (Yamashita *et al*., 1997).

As mentioned before, 20:4 is also incorporated into the PLs of biofilms formed by these strains. In *C. albicans* CBS 562T (Fig. 11a) and *C. dubliniensis* NRRL Y-17841T (Fig. 11c) 20:4 is incorporated into all the PL fractions. In these strains the concentration 20:4 remained constant in the PL fractions, except for the PI fraction, where there was a decrease in the concentration 20:4 over time. In *C. dubliniensis* NRRL Y-17841T no 20:4 was present in the PI fraction after 48 h. A possible explanation for the observed results might be that phospholipases cleave 20:4 from PI and that it is moved between the different PL fractions, through deacylation – reacylation steps (Fig. 10), to maintain

homeostasis in the membrane (Pérez *et al*., 2006). Arachidonic acid in PI might also be used for other processes, such as eicosanoid production, since it is known that 20:4 is cleaved from mammalian PI in order to produce eicosanoids (Hong & Deykin, 1981). In *C. albicans* NRRL Y-0477 (Fig. 11b), 20:4 was not incorporated into all the PL fractions. It appears that the 20:4 is moved between the different phospholipid fractions during biofilm formation (Pérez *et al*., 2006) or that different phospholipid biosynthetic pathways (Fig. 9) dominate at different times during biofilm formation. However, a similar pattern is observed for the PI fraction, with a decrease in the concentration 20:4 over time. This might also be explained by the possible production of 20:4 metabolites (Hong & Deykin, 1981).

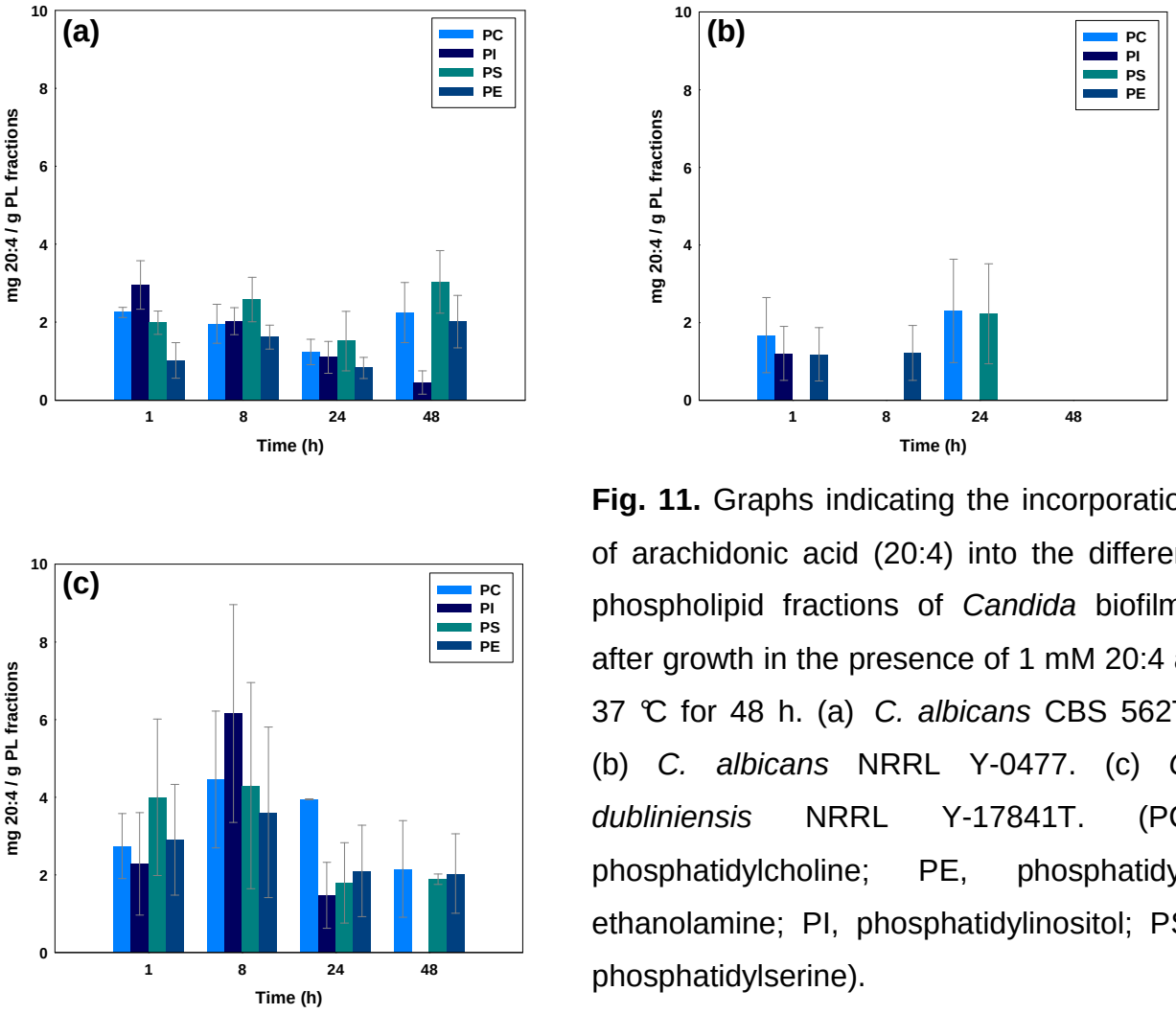


Fig. 11. Graphs indicating the incorporation of arachidonic acid (20:4) into the different phospholipid fractions of *Candida* biofilms after growth in the presence of 1 mM 20:4 at 37 ℃ for 48 h. (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-0477. (c) *C. dubliniensis* NRRL Y-17841T. (PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine).

Membrane homeostasis is also influenced by the PE/PC ratio of cell membranes. Phosphatidylcholine is a cylindrical shaped molecule and has a tendency to form lipid bilayers, while PE is a conical shaped molecule with a relatively small head group coupled to a relatively large area occupied by acyl chains (Williams, 1998). This allows for the tendency of PE to form nonlamellar structures. These two physical forms of the major PLs are tightly regulated in order to provide the optimum curvature of the cell membrane (de Kroon, 2007). Interestingly, the degree to which PE assumes a conical shape is determined by the acyl chain length and unsaturation.

From the obtained results it is clear that there were similar changes in the PE/PC ratios of *C. albicans* CBS 562T and *C. dubliniensis* NRRL Y-17841T (Fig. 12a, c) in the presence of 20:4. For *C. albicans* CBS 562T there was a significant increase in the PE/PC ratio during the early phase of biofilm formation in the presence of 20:4, compared to the PE/PC ratio in the absence of 20:4. Incorporation of long chain polyunsaturated fatty acids (PUFAs) such as 20:4, into the PE fraction, will result in an increase in the non-bilayer propensity (conical shape) of the PE molecule (de Kroon, 2007). In literature, growth of *Saccharomyces cerevisiae* on exogenous long-chain PUFAs led to a decrease in the PE/PC ratio in order to counteract this destabilising effect. However, PE molecules can form intermolecular hydrogen bonds, leading to an increased membrane order (Williams, 1998), and an increase in PE/PC ratio in a cell membrane may force the PE molecules into a more cylindrical shape (de Kroon, 2007), resulting in a reduction in the non-lipid bilayer propensity of PE.

Interestingly, during the intermediate and mature phase this ratio decreased to similar levels as in the absence of 20:4. This is similar to the findings of Janssen *et al.* (2000), who found a pronounced decrease in the PE/PC ratio when *S. cerevisiae*, grown on a non fermentable carbon source, entered the stationary phase.

In *C. albicans* NRRL Y-0477 (Fig. 12b), no significant difference in the PE/PC ratio in the presence and absence of 20:4 was found. This correlates with the lack of 20:4 incorporation into the PLs of this strain and the observed stability of the unsaturation index of the PLs, rendering such a compensation mechanism unnecessary.

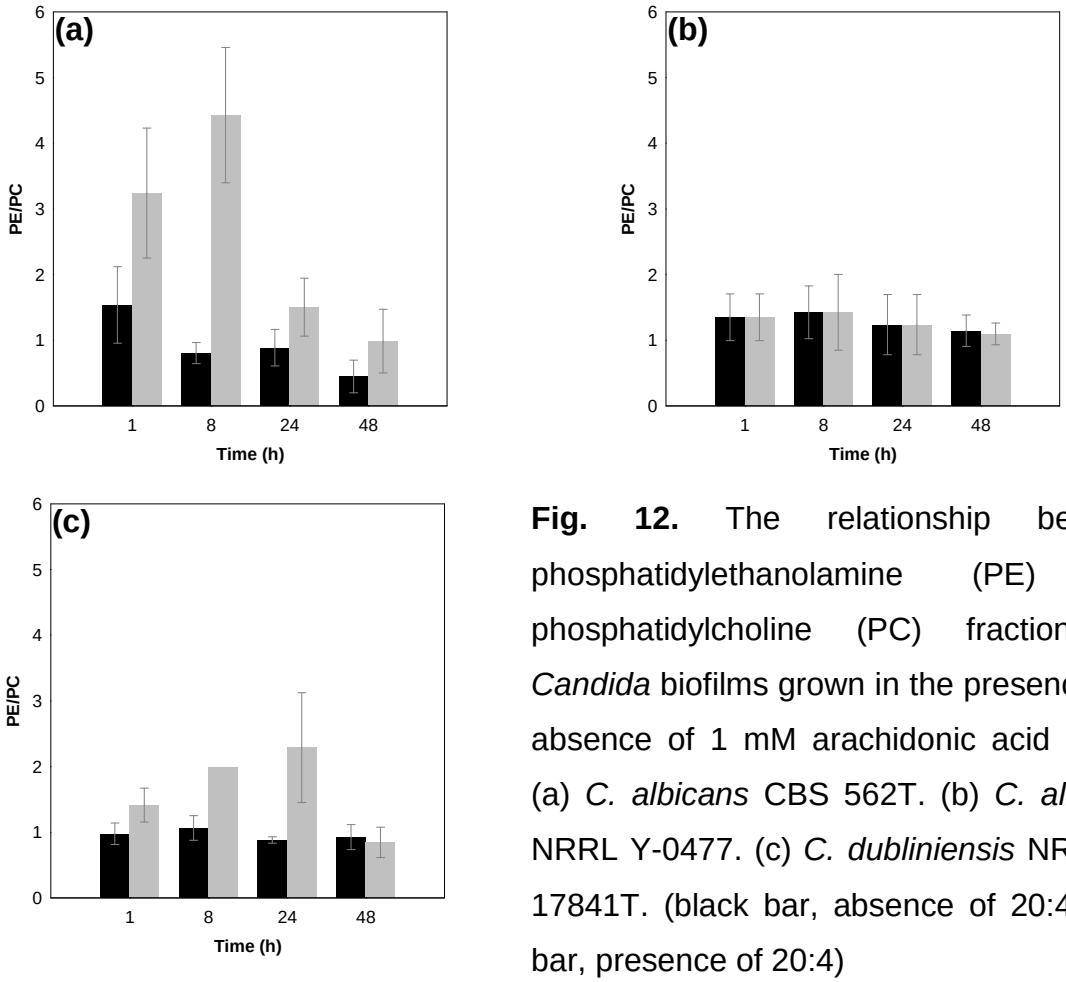


Fig. 12. The relationship between phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions of *Candida* biofilms grown in the presence and absence of 1 mM arachidonic acid (20:4). (a) *C. albicans* CBS 562T. (b) *C. albicans* NRRL Y-0477. (c) *C. dubliniensis* NRRL Y-17841T. (black bar, absence of 20:4; grey bar, presence of 20:4)

4.4.6) Eicosanoid production

The oxygenated metabolites of twenty carbon PUFAs (e.g. 20:4, eicosapentaenoic acid, eicosatrienoic acid) are known as eicosanoids (Erb-Downward & Huffnagle, 2006). Examples of eicosanoids include the prostanoids (prostaglandins, thromboxanes), produced via cyclooxygenases (COX) (Brash, 2001, Noverr *et al*., 2003), the leukotrienes, lipoxins and hydroxy fatty acids produced via lipoxygenases (Noverr *et al*., 2003) as well as 3-hydroxy (3-OH) fatty acids produced via β-oxidation (incomplete β-oxidation) as well as by cytochrome P450 enzymes (Brash, 2001; Venter *et al*., 1997).

Although eicosanoid production has been well studied in mammalian cells, little is known about the pathways involved in eicosanoid production by fungi. Tsitsigiannis *et al*. (2005a) identified three COX-like dioxygenase enzymes, which are structurally similar to mammalian COX, encoded by *ppoA*, *ppoB* and *ppoC* genes, in the opportunistic pathogens *Aspergillus nidulans* and *A.*

fumigatus. These dioxygenases contain amino acid sequences with both oxygenase and peroxidase regions and are responsible for the production of oxylipins from oleic and linoleic acid (Tsitsigiannis *et al*., 2005a; b). Noverr *et al*. (2001), concluded that the pathogenic yeasts *C. albicans* and *Cryptococcus neoformans* have the ability to produce and secrete prostaglandins (PGE_2) *de novo* or from 20:4 and speculated that COX-like enzymes must be present in these yeasts. Erb-Downward & Noverr (2007) subsequently identified the enzymes responsible for prostaglandin production in *C. albicans*, as a desaturase homolog (Ole2) and a multicopper oxidase homolog (Fet3). This indicates that *C. albicans* has novel pathways for the production of eicosanoids. In addition, Venter *et al*. (1997) indicated that 3-OH fatty acids are produced in *Dipodascopsis uninucleata* through incomplete β-oxidation and Deva *et al*. (2000) indicated that *C. albicans* was capable of producing 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18-diHETE) from 20:4, but did not speculate on the metabolic pathway involved. They found that this compound was localised in hyphae but not in yeast cells.

The present study confirms the ability of mature *C. albicans* CBS 562T biofilms to produce 3,18-diHETE. Figure 13 is a mass spectrum of the compound with a retention time of 26 min. This compound had a major ion with an *m/z* of 175, which is characteristic of a 3-OH fatty acid (hydroxylation at carbon 3), as well as an ion with an *m/z* of 131, indicating hydroxylation at carbon 18 (Deva *et al*., 2000). The mother ion with an *m/z* of 494 is also present. The same spectra were obtained for *C. albicans* NRRL Y-0477 and *C. dubliniensis* NRRL Y-17841T. This is the first report of the production of an eicosanoid by *C. dubliniensis*. None of the strains produced 3,18-diHETE in the absence of 20:4. Interestingly, we found that *C. dubliniensis* NRRL Y-17841T biofilm was capable of producing a second 3-OH fatty acid in the presence of 20:4. This compound had a retention time of 36 min, but still needs to be identified.

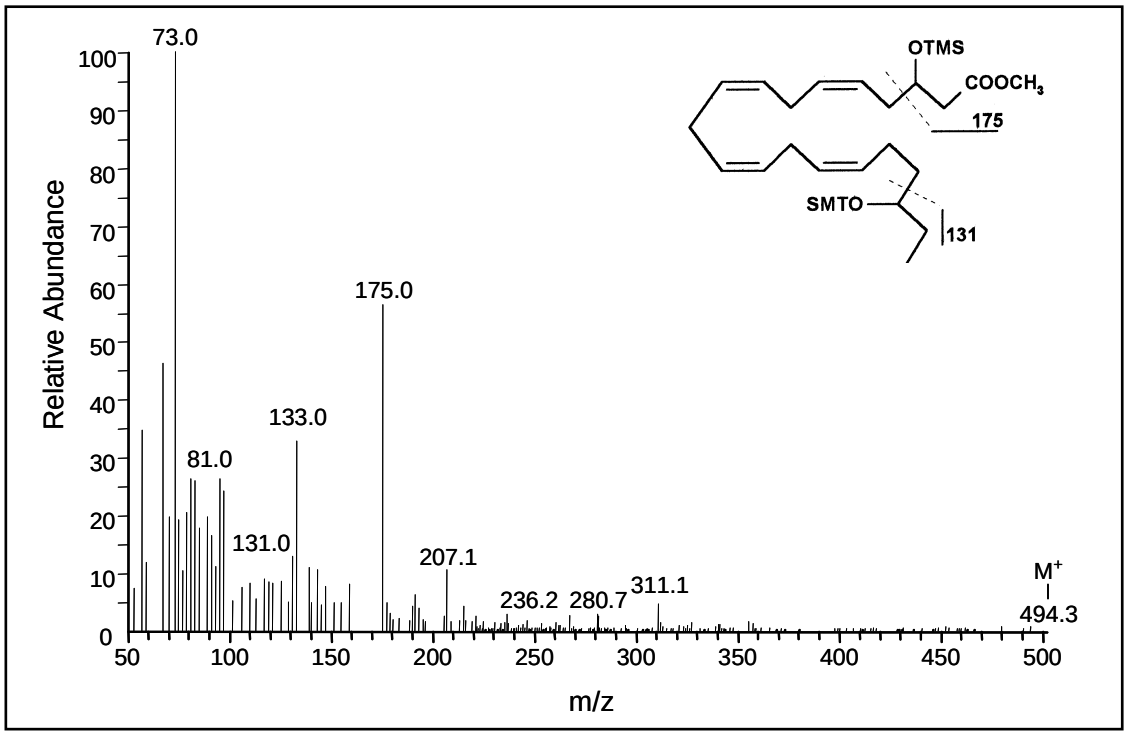


Fig. 13. The mass spectrum of 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18 di-HETE) with a retention time of 26 min. This compound was produced by biofilms of *Candida albicans* and *C. dubliniensis* strains in the presence of arachidonic acid (20:4).

4.5) Conclusions

The study of lipids and their metabolites are becoming increasingly important in the pathogenesis of fungi, either as virulence factors or as potential carbon source. As can be seen from the results, yeasts have complex mechanisms for regulating the metabolism of lipids and pathways for synthesising bioactive metabolites. It was found that planktonic *Candida* strains are capable of taking up exogenous 20:4. However, differences in uptake mechanisms between the *Candida* strains were observed, with *C. albicans* strains exhibiting probably an unsaturable uptake mechanism and *C. dubliniensis* a saturable uptake mechanism. The 20:4 that is taken up was probably used for cellular metabolism, which may include β-oxidation or eicosanoid production, as well as incorporated into the NLs and GLs of these three strains. Variation was observed between strains regarding the amount of 20:4 used for cellular

metabolism and incorporation into the different lipid fractions. Interestingly no 20:4 was found in the PL fractions of these planktonic strains.

The biofilms formed by these strains were also capable of taking up exogenous 20:4. Differences in the uptake were again observed, with *C. albicans* CBS 562T and *C. dubliniensis* NRRL Y-17841T biofilms exhibiting the same uptake mechanism as the planktonic cells, but *C. albicans* NRRL Y-0477 biofilms possibly having a different uptake mechanism than the planktonic cells. Arachidonic acid was also incorporated into the NLs and GLs of the biofilms, as well as into the different PL fractions of all three strains. In all these *Candida* biofilms there was a decrease in the 20:4 in the PI fraction during biofilm formation. It is postulated that PL remodelling might be involved in these biofilms or that the 20:4 is released from the PI fraction for the production of eicosanoids. It was found that *C. albicans* and *C. dubliniensis* produced 3,18 di-HETE from 20:4. This is the first time this compound is found in *C. dubliniensis*. In addition, *C. dubliniensis* was capable of producing a novel 3-OH fatty acid from 20:4.

The observed influence of 20:4 on the unsaturation of these *Candida* biofilm membranes could affect their susceptibility towards antimicrobials (Hąc-Wydro *et al.*, 2007; Yamaguchi, 1977) and is the subject of the next chapter.

4.6) References

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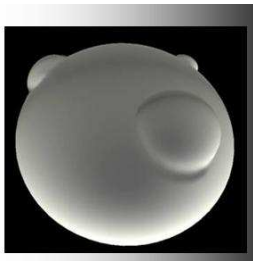
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CHAPTER 5

Arachidonic acid increases antifungal susceptibility of *Candida albicans* and *C. dubliniensis* biofilms

Parts of chapter published in Journal of Antimicrobial Chemotherapy 63, 124–128, (2009).

5.1) Abstract

During *Candida albicans* infection arachidonic acid (20:4) is released from the phospholipids (PLs) of the infected host cell membrane and used by *C. albicans* as sole carbon source and for the production of eicosanoids. In addition, 20:4 can be incorporated into the PLs of yeasts, influencing the level of saturation in yeast cell membranes. It is suggested that the effectiveness of polyene (e.g. amphotericin B) and imidazole (e.g. clotrimazole) antifungals may depend upon the level of unsaturation and ergosterol content of the membrane. Therefore, the aim of this study was to evaluate the effect of 20:4 on the cell membrane and susceptibility of *C. albicans* and *C. dubliniensis* biofilms towards amphotericin B and clotrimazole. *Candida albicans* and *C. dubliniensis* biofilms were grown in the presence and absence of 20:4 and the effect of amphotericin B and clotrimazole examined by confocal laser scanning microscopy, determination of mitochondrial metabolism, unsaturation index of the PL fractions and ergosterol content of the membranes. Arachidonic acid had no effect on the viability of the cells in the biofilm; but influenced PL unsaturation and ergosterol content of both *C. albicans* and *C. dubliniensis* type strains, increasing susceptibility to the antifungals. Pre-treatment of biofilms with polyunsaturated fatty acids (PUFAs) may result in the reduction in antifungal dose needed to inhibit biofilms.

5.2) Introduction

During *Candida albicans* infection, 20:4 is released from the PLs of infected host cell membranes by fungal phosholipases (Deva *et al*., 2001). *Candida albicans* has the ability to utilise 20:4 as sole carbon source to stimulate cell growth and morphogenesis. Arachidonic acid is also a precursor for the production of bioactive eicosanoids which are known as important virulence factors, stimulating germ tube formation and inflammation during infection (Alem & Douglas, 2004; Erb-Downward & Noverr, 2007). It has also been reported that 20:4 can be taken up and incorporated into the phospholipids of yeasts, influencing the level of unsaturation in yeast cell membranes (Kock & Ratledge, 1993). This was also shown in the previous chapter, where 20:4 uptake and incorporation by these strains were evaluated.

Two classes of membrane active antifungals are commonly used to treat *Candida* infections: the polyenes, e.g. amphotericin B, which bind to ergosterol in fungal cell membranes, causing membrane disruption, and the azoles, e.g. clotrimazole, an ergosterol synthesis inhibitor, which increase cellular permeability (Graybill, 2000). Hąc-Wydro *et al.* (2007) suggested that nystatin, a polyene drug, may bind more strongly to monolayers of PLs, containing two unsaturated fatty acids than to monolayers of saturated PLs with similar acyl chain length. Similarly, Yamaguchi (1977) found that imidazole antifungals interact with unsaturated PLs extracted from *C. albicans* protoplast membranes as well as free PUFAs (including 20:4), and that the presence of unsaturated PLs influenced the sensitivity of liposomes towards imidazole antifungals (Yamaguchi & Iwata, 1979).

Since 20:4 is present in the natural habitat of infectious *C. albicans* and plays an important role during infection, the aim of this study was to investigate the effect of this PUFA on the susceptibility of selected strains of *C. albicans* and *C. dubliniensis* biofilms towards amphotericin B and clotrimazole.

5.3) Materials and methods

5.3.1) Strains used

The following strains were used in this study: *Candida albicans* CBS 562T, *C. albicans* NRRL Y-0477, *C. dubliniensis* NRRL Y-17841T. The strains were maintained on yeast malt extract (YM) agar [10 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone, 16 g agar, 1000 mL distilled water (dH_20)] at room temperature.

5.3.2) XTT antifungal susceptibility assay

The strains, from 24 h old cultures on YM agar plates, were grown in 20 mL sterile yeast nitrogen base (YNB) broth [6.7 g YNB, 1000 mL dH_20] containing 10 $g.L^{-1}$ glucose, at 30 ℃ for 48 h in sterile centrifuge tubes. The cells were harvested by centrifugation for 10 min at 4000 *g*, washed with sterile phosphate buffered saline (PBS) (OXOID, UK) and resuspended in 10 mL filter sterilised RPMI-1640 medium (Sigma-Aldrich, USA). The cells were counted with a hemacytometer and diluted to 1 x 10^6 $cells.mL^{-1}$ in filter

sterilised RPMI-1640 medium. Arachidonic acid (Sigma-Aldrich, USA) (in ethanol) was added to this suspension to a final concentration of 1 mM and 100 µL were dispensed into the wells of a 96-well microtiter plate (Corning Incorporated, Costar®, USA) and incubated at 37 ℃ for 48 h in order to allow biofilm formation (Ramage *et al*., 2001). Appropriate controls were included. The antifungal susceptibility assay was performed according to Al-Fattani & Douglas (2006) and Bachmann *et al*. (2002). Stock solutions (50 $g.L^{-1}$) of amphotericin B (Sigma-Aldrich, USA) and clotrimazole (Sigma-Aldrich, USA) were prepared in dimethylsulfoxide (Sigma-Aldrich, USA), diluted in the growth medium, buffered to pH 7.4 with 0.165 M MOPS (Sigma-Aldrich, USA), and used immediately. Hundred microlitres of the diluted antifungals were added to the biofilms and the plates incubated for another 5 h at 37 ℃. Fifty microlitres of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl]-2H tetrazolium hydroxide (XTT) (Sigma-Aldrich, USA) (0.5 g XTT in 1000 mL Ringer's lactate solution, filter sterilised, aliquoted and stored at -70 ℃) and 4 µL of 1 mM menadione (Fluka, USA) (10 mM menadione in acetone, further diluted to 1 mM) were added into each well and the plates were incubated in the dark at 37 ℃ for 3 h. Subsequently the optical density was measured at 492 nm on a Labsystems iEMS Reader (Thermo Bioanalysis, Helsinki, Finland). The results were interpreted as the percentage inhibition caused by the antifungals on the biofilms grown in the presence of 20:4 compared to biofilms grown in the absence of 20:4.

5.3.3) Visualisation of antifungal susceptibility

Biofilms of each strain were formed in chamber slides (Lab-Tek® Chamber Slide™ System, USA) containing 3 mL filter sterilised RPMI-1640 media for 48 h at 37 ℃ in the presence and absence of 1 mM 20:4 . Amphotericin B (12.5 $g.L^{-1}$) and clotrimazole (1.25 $g.L^{-1}$), diluted in buffered (pH 7.4) growth medium, were added and the biofilms were incubated for another 5 h at 37 ℃ with appropriate controls. Biofilms were stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit (Invitrogen, Molecular Probes, USA) and visualised using a confocal laser scanning microscope (CLSM) (Jin *et al*., 2005).

5.3.4) Ergosterol content

Biofilms of each strain were formed in polystyrene Petri dishes containing filter sterilised RPMI-1640 media for 48 h at 37 ℃. Washe d biofilms were scraped off and placed into glass borosilicate tubes (Schott, USA) and the wet weight of the cells determined. Extraction of ergosterol was according to Arthington-Skaggs *et al*. (2000) with the following modifications: Potassium hydroxide (MERCK, Germany), dissolved in methanol/ethanol/water (700 : 315 : 15, by vol.) was added to the cells. A further 2 mL ethanol was added and the tubes gassed with nitrogen gas. The tubes were incubated in a water bath at 80 ℃ for 90 min and shaken every 10 min. Sterols were extracted with *n*-heptane (Burdick & Jackson, USA) and stored at 4 ℃. The he ptane fraction was dried under nitrogen gas and dissolved in 2 ml ethanol. Solids were precipitated overnight at 4 ℃. The samples were filtered (0.45 µm) and the absorbance measured at 282 nm on a SpectraMax M2 Microplate Reader (Molecular devices, USA). This was performed in duplicate.

5.3.5) Statistical analysis

Unless stated otherwise, all experiments were performed at least in triplicate. The *t*-test was performed to determine if the data sets obtained were significantly different from each other with a P-value equal to or less than 0.05.

5.4) Results and discussion

5.4.1) XTT antifungal susceptibility assay

The results of this assay (Fig. 1) clearly indicate an increase in susceptibility of *C. albicans* CBS 562T biofilms towards both amphotericin B (Fig. 1a) and clotrimazole (Fig. 1b) after growth in the presence of 1 mM 20:4, compared to the susceptibility of biofilms grown in the absence of 20:4. The same effect of growth in the presence of 20:4 was not as profound for *C. albicans* NRRL Y-0477. No statistically significant increase in inhibition was observed for clotrimazole (Fig. 1d) and only at high amphotericin B concentrations did the biofilms grown in the presence of 20:4 exhibit increased susceptibility (Fig. 1c). *Candida dubliniensis* NRRL Y-17841T biofilms also exhibited a significant increase in susceptibility at all amphotericin B concentrations tested (Fig. 1e),

as well as towards clotrimazole at concentrations ranging from 0.1 $mg.L^{-1}$ to 50 $mg.L^{-1}$ (Fig. 1f).

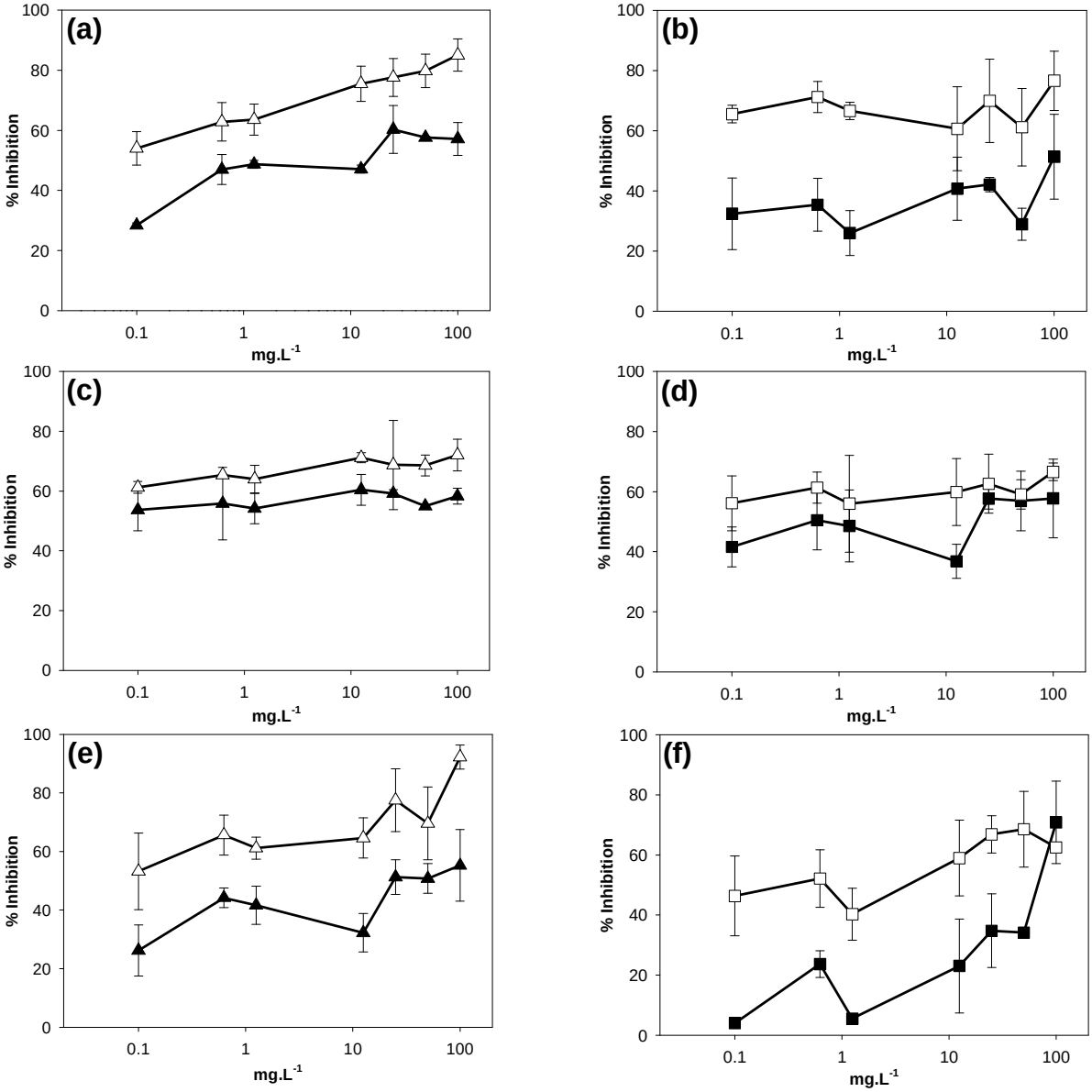


Fig. 1. Percentage inhibition of antifungals on the mitochondrial metabolism of biofilms formed by *Candida* species grown in the presence and absence of arachidonic acid (20:4). (a, b) *C. albicans* CBS 562T, (c, d) *C. albicans* NRRL Y-0477, (e, f) *C. dubliniensis* NRRL Y-17841T.

(Δ, biofilms grown in the presence of 20:4, treated with amphotericin B. ▲, biofilms grown in the absence of 20:4, treated with amphotericin B. □, biofilms grown in the presence of 20:4, treated with clotrimazole. ■, biofilms grown in the absence of 20:4, treated with clotrimazole)

5.4.2) Visualisation of antifungal susceptibility

Since discrepancies have been reported between results obtained for XTT assays and actual biofilm formation measured as dry weight, indicating a lack of correlation between metabolism and biofilm formation (Kuhn *et al*., 2003), it was decided to visualise the effect of growth in the presence of 20:4 on antifungal susceptibility, microscopically. As indicated in Chapter 3 (Fig. 5), mature biofilms formed by the *Candida* strains in the absence of 20:4, stained with the viability kit, contained a proportion of dead cells. This might be due to nutrient or oxygen starvation as a result of the increasing biomass. When these untreated controls were compared to mature biofilms formed in the presence of 1 mM 20:4, no difference could be observed for all three strains, indicating that 1 mM 20:4 has no influence on the viability of the biofilms.

As expected, an increase in dead cells was observed when biofilms of *C. albicans* CBS 562T (Fig. 2c) and *C. dubliniensis* NRRL Y-17841T (Fig. 4c), grown in the presence of 1 mM 20:4, were treated with amphotericin B, compared to biofilms grown in the absence of 20:4 and treated with amphotericin B (Fig. 2a, 4a). Interestingly, this was also found for *C. albicans* NRRL Y-0477 (Fig. 3a, c) even though the results of the XTT assay showed no significant differences. An increase in dead cells can also be observed after treatment of biofilms, grown in the presence of 20:4, with clotrimazole (Fig. 2d, 3d, 4d), compared to biofilms grown in the absence of 20:4 and treated with clotrimazole (Fig. 2b, 3b, 4b). This confirms that growth in the presence of 1 mM 20:4 renders the tested *Candida* strains more susceptible to amphotericin B and clotrimazole although there are strain specific variations as observed for *C. albicans*.

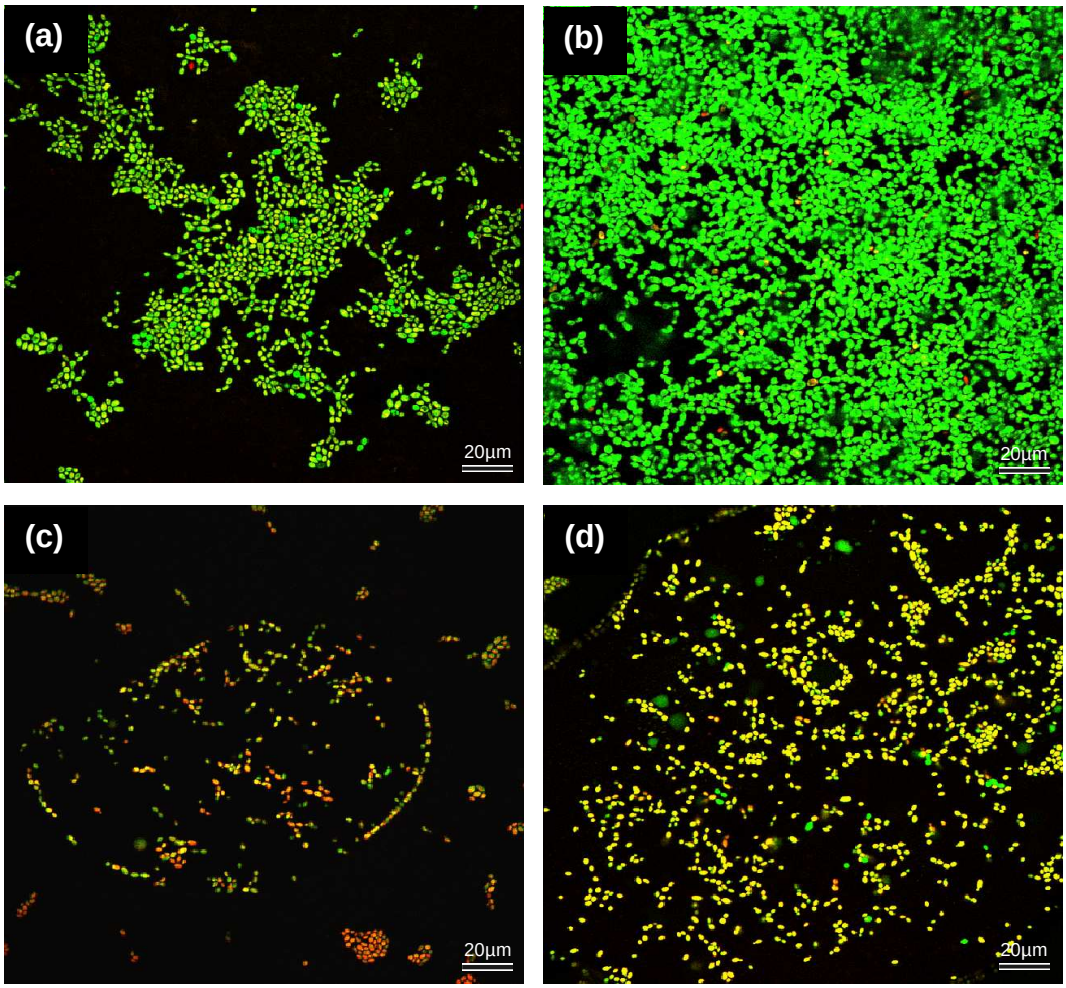


Fig. 2. Confocal laser scanning micrographs of *Candida albicans* CBS 562T biofilms. (a) Biofilms grown in the absence of arachidonic acid (20:4) treated with amphotericin B. (b) Biofilms grown in the absence of arachidonic acid (20:4) treated with clotrimazole. (c) Biofilms grown in the presence of 20:4, treated with amphotericin B. (d) Biofilms grown in the presence of 20:4, treated with clotrimazole. Green fluorescence indicates live cells and red fluorescence, dead cells.

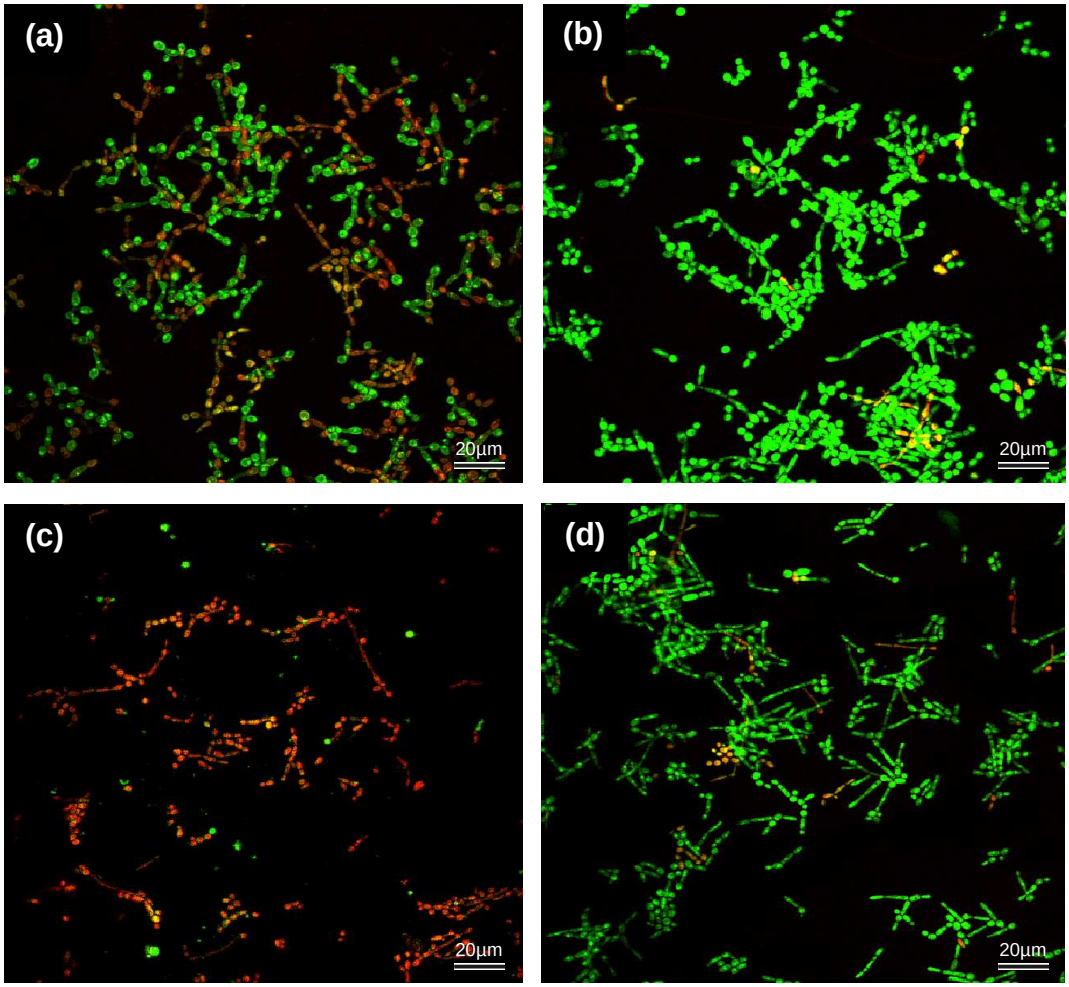


Fig. 3. Confocal laser scanning micrographs of *Candida albicans* NRRL Y-0477 biofilms. (a) Biofilms grown in the absence of arachidonic acid (20:4) treated with amphotericin B. (b) Biofilms grown in the absence of arachidonic acid (20:4) treated with clotrimazole. (c) Biofilms grown in the presence of 20:4, treated with amphotericin B. (d) Biofilms grown in the presence of 20:4, treated with clotrimazole. Green fluorescence indicates live cells and red fluorescence, dead cells.

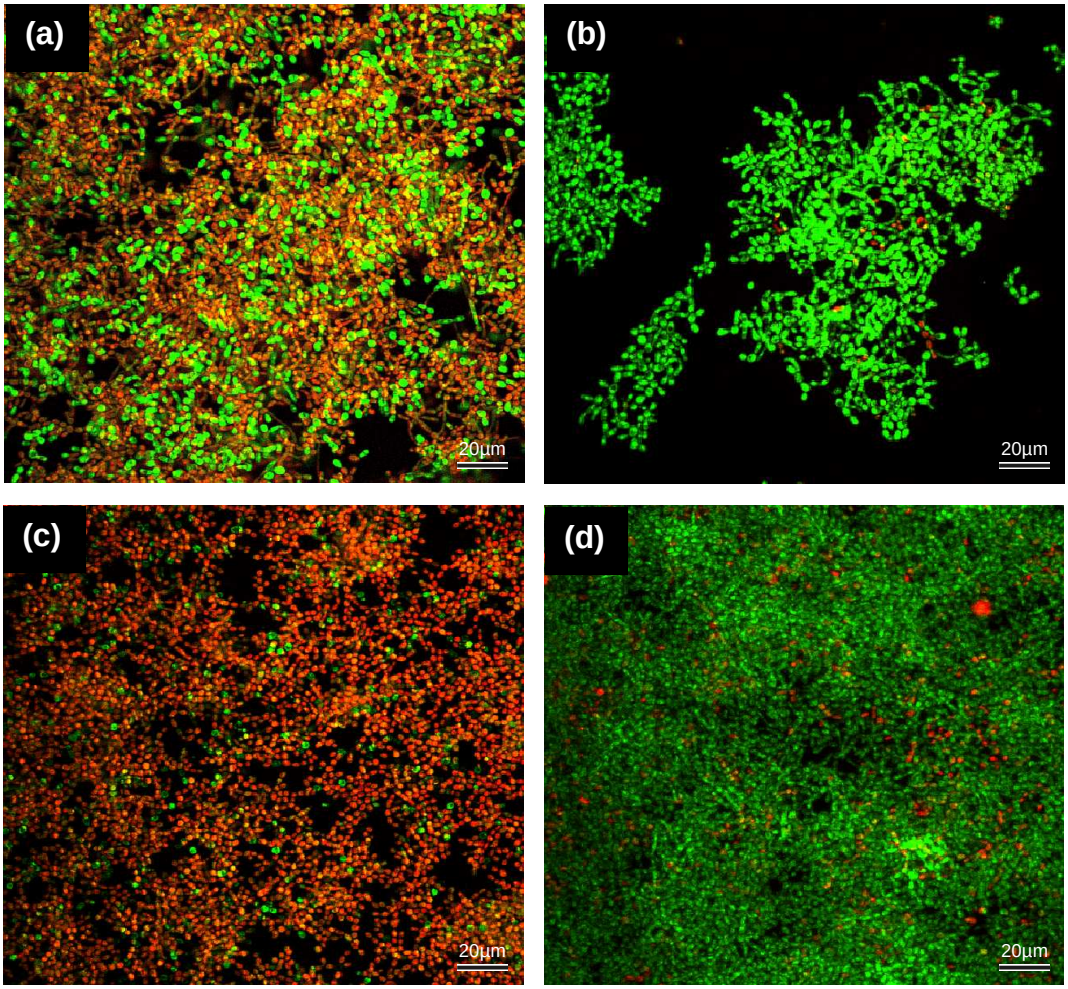


Fig. 4. Confocal laser scanning micrographs of *Candida dubliniensis* NRRL Y-17841T biofilms. (a) Biofilms grown in the absence of arachidonic acid (20:4) treated with amphotericin B. (b) Biofilms grown in the absence of arachidonic acid (20:4) treated with clotrimazole. (c) Biofilms grown in the presence of 20:4, treated with amphotericin B. (d) Biofilms grown in the presence of 20:4, treated with clotrimazole. Green fluorescence indicates live cells and red fluorescence, dead cells.

5.4.3) Ergosterol content

As mentioned previously, ergosterol is the main target for the action of many antifungals, including amphotericin B (Graybill, 2000). The ergosterol content of both the type strains increased significantly in biofilms grown in the presence of 20:4, i.e. for *C. albicans* CBS 562T from 629.1 $mg.g^{-1}$ (± 24.6) to 680.1 $mg.g^{-1}$ (± 4.1) and for *C. dubliniensis* NRRL Y-17841T from 303.0 $mg.g^{-1}$ (± 4.9) to 340.8 $mg.g^{-1}$ (± 27.4). However, for *C. albicans* NRRL Y-0477 there

was a significant decrease in ergosterol content from 136.4 $mg.g^{-1}$ (± 0.4) to 91.6 $mg.g^{-1}$ (± 5.5) in the presence of 20:4.

It is known that yeasts (e.g. *Saccharomyces cerevisiae*) can react to the uptake of exogenous fatty acids by altering their PLs and in some strains the ergosterol content (McDonough *et al*., 2002). Similar results were obtained in this study. The increase in ergosterol content of *C. albicans* CBS 562T and *C. dubliniensis* NRRL Y-17841T grown in the presence of 20:4, may explain the increase in amphotericin B susceptibility of these strains, however the mechanism behind increased clotrimazole susceptibility is unclear. The reason for the decrease in ergosterol content for *C. albicans* NRRL Y-0477 in the presence of 20:4 is unknown.

5.4.4) Phospholipid composition and unsaturation in membranes

The fatty acid profiles of the major PL fractions, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), of these strains after 48 h of growth were also determined from data obtained in Chapter 4. In all three strains there were no significant difference in the fatty acid profiles of the PE fraction in the presence and absence of 20:4, except for the increase in 20:4 in this fraction of *C. albicans* CBS 562T [1.6 (± 0.0)] and *C. dubliniensis* NRRL Y-17841T [2.4 (± 0.4)], and with no 20:4 present in *C. albicans* NRRL Y-0477. However, there was no significant difference in the unsaturation index for the PE fractions of these strains.

Interestingly, the PC fraction of *C. albicans* CBS 562T biofilms grown in the presence of 20:4, showed an increase in the percentage polyunsaturated linoleic acid (18:2), [from 5.7 (± 1.6) to 11.0 (± 1.5)] and a decrease in the percentage saturated palmitic acid (16:0) [from 42.7 (± 2.7) to 34.3 (± 4.2)] and stearic acid (18:0) [from 17.9 (± 1.9) to 9.7 (± 1.7)]. The PC fraction of *C. dubliniensis* NRRL Y-17841T biofilms grown in the presence of 20:4, also showed an increase in the percentage 18:2 [2.9 (± 4.8) to 11.4 (± 1.8)] and a decrease in the percentage 16:0 [from 50.5 (± 1.9) to 38.8 (± 1.3)]. This, taken together with an increase in 20:4 in the PC fraction [*C. albicans* CBS 562T: 1.9 (± 0.5); *C. dubliniensis* NRRL Y-17841T: 5.1 (± 1.1)] contributed to the increase in unsaturation index of this fraction from 0.4 (± 0.0) to 0.7 (± 0.1) for

C. albicans CBS 562T and from 0.3 (± 0.1) to 0.7 (± 0.1) for *C. dubliniensis* NRRL Y-17841T.

In the case of *C. albicans* NRRL Y-0477, no statistically significant differences in fatty acid profiles could be observed for the PC fraction. This, taken together with the absence of 20:4 in the PL fractions after 48 h (Chapter 4, Fig. 11b) contributed to the lack of significant difference in unsaturation index of the PL fractions of this strain.

Membrane saturation may play an important role in the effectiveness of antifungal therapy (Hąc-Wydro *et al*., 2007; Yamaguchi, 1977; Yamaguchi & Iwata, 1979). The calculated unsaturation indices clearly indicate that there is an increase in the unsaturation of the PLs in the cell membrane of biofilms of the type strains, grown in the presence of 20:4. This increase may affect membrane fluidity and membrane protein function possibly allowing increased uptake of antifungals, such as clotrimazole (McDonough *et al*., 2002). The significant increase in unsaturation was only observed for *C. albicans* CBS 562T and *C. dubliniensis* NRRL Y-17841T and correlates with the greater susceptibility towards antifungals exhibited by these two strains. The absence of change in unsaturation of the PLs in the cell membrane of *C. albicans* NRRL Y-0477 correlates with the absence in increased antifungal susceptibility (as measured by the XTT assay) of this strain, when grown in the presence of 20:4. Interestingly similar results were reported by Mehta (1983), when they combined aculeacin A, which is a beta-glucan inhibitor, with fatty acids, such as lauric acid, palmitoleic acid, palmitelaidic acid, oleic acid, 18:2, linolenic acid and 20:4. They found an increase in the susceptibility towards aculeacin A when combined with these fatty acids, but no effect with the saturated fatty acids, 16:0 and 18:0. In addition, Goyal & Khuller (1994) found that *C. albicans* mycelial forms, containing more unsaturated PLs than yeast forms, were more susceptible to amphotericin B, nystatin and miconazole.

In contrast to these findings, Peyron *et al*. (2002) indicated that there is no correlation between PL fatty acid composition and amphotericin B resistance in *Candida lusitania*. Furthermore, Kohli *et al*. (2002) and Mishra *et al.* (2008) found that fluconazole resistant *C. albicans* strains had an increased membrane fluidity compared to the sensitive strains. Younsi *et al*. (2000) also found that amphotericin B resistant strains of *Kluyveromyces lactis* had an

increased membrane fluidity. In addition, it was indicated by Iannitelli & Ikawa (1980) that the effectiveness of amphotericin B on *S. cerevisiae* is decreased in the presence of PUFAs with increasing chain length, including 20:4. However, this may be explained by the antagonistic interaction between the antifungal and unsaturated free fatty acids simultaneously present in the medium, as reported for imidazole antifungals (Yamaguchi, 1977) and amphotericin methyl esters (Gale *et al*., 1975).

An alternative or complementary mechanism may be the increased oxidative stress placed on the organisms incorporating PUFAs into their cellular lipids. According to Krasowska *et al*. (2007), PUFAs are easily oxidized to produce free radicals, increasing the oxidative stress of an organism. From this it can be inferred that an increase in intracellular 20:4 may lead to an increase in oxidative stress. Interestingly, Fekete *et al*. (2007) indicated that oxidative stress may change the susceptibility of *C. albicans* towards antifungals, including amphotericin B, and that more susceptible strains contained more intracellular PUFAs.

Since there is a need to develop efficient new drug therapies for the treatment of mycoses (Fekete *et al*., 2007), pre-treatment of biofilms with 20:4 and/or other long-chain PUFAs may result in the reduction of the amount of antifungal needed to inhibit *Candida* biofilms, leading to lowered toxicities and economical advantages.

5.5) Conclusions

In this study it was indicated that when *C. albicans* and *C. dubliniensis* biofilms were grown in the presence of 20:4, there was an increase in susceptibility towards amphotericin B and clotrimazole. However, variations between strains were observed. The reasons behind this have been postulated to involve the increase in fluidity of the membranes, due to the incorporation of a PUFA (20:4) in the PLs, the change in the ergosterol content and/or the increase in oxidative stress. Even though similar results have been obtained by other researchers, the opposite has also been found, highlighting the importance of establishing the conserved status of this phenomenon as well as the optimum time of exposure to PUFAs prior to antifungal treatment.

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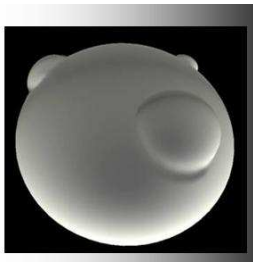
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SUMMARY / OPSOMMING

Summary
Candida albicans and *C. dubliniensis* are two closely related pathogenic yeast species, sharing many phenotypic characteristics which make it difficult to differentiate them, especially in clinical samples. As a result of the similarities between these species, identification techniques, based on phenotypic characteristics, have been developed. In this study some of these techniques and virulence factors were used to characterise strains belonging to these species and to select phenotypically dissimilar strains for further study. This was performed to evaluate if the effect of arachidonic acid (20:4) on these strains were the same, even though they are phenotypically different. *Candida albicans* and *C. dubliniensis* can form biofilms which play an important role during infection. During *C. albicans* infection, 20:4, a long-chain polyunsaturated fatty acid (PUFA), derived from the phospholipids (PLs) of the infected host cell membrane, serves as carbon source and precursor for eicosanoid production. Conflicting results are presented in literature regarding the effect of 20:4 on morphogenesis in *C. albicans*. In addition, the effect of 20:4 on growth and morphology of *C. dubliniensis* is unknown. Microscopic examination and enzyme activity assay indicated that 1 mM 20:4 had little to no effect on growth and metabolic activity of planktonic cells and biofilms, as well as on the morphology and viability of the cells in the biofilms. The uptake of PUFAs by yeasts is necessary for utilisation as metabolic fuels, cellular building blocks and the production of signalling molecules. However, there are no definitive studies regarding the uptake and cellular metabolism of 20:4 by these pathogenic yeasts. The uptake and incorporation of 20:4 by planktonic cells and biofilms of selected strains of *C. albicans* and *C. dubliniensis* were evaluated by subjecting residual and cellular lipids from planktonic cells and biofilms, grown in the presence and absence of 20:4, to gas chromatography and gas chromatography-mass spectrometry. Strain specific variation in 20:4 uptake and incorporation into different lipid fractions of planktonic cells and biofilms were found. In addition, eicosanoids produced by biofilms in the presence of 20:4 were extracted and it was found that biofilms of these strains were capable of producing 3-hydroxy fatty acids from 20:4. Arachidonic acid can be incorporated into the PLs of yeasts, influencing saturation in cell membranes. It is suggested that the effectiveness of antifungals may depend

upon the level of unsaturation and ergosterol content of the membrane, therefore the effect of 20:4 on the cell membrane and susceptibility of *C. albicans* and *C. dubliniensis* biofilms towards amphotericin B and clotrimazole were also determined. This was performed by confocal laser scanning microscopy, determination of mitochondrial metabolism, unsaturation index of the PL fractions and ergosterol content of the membranes of biofilms grown in the presence and absence of 20:4. The results indicated that 20:4 influences PL unsaturation and ergosterol content of both *C. albicans* and *C. dubliniensis* type strains, increasing susceptibility to the antifungals. Pre-treatment of biofilms with PUFAs may result in the reduction in antifungal dose needed to inhibit biofilms.

Keywords:
Amphotericin B, Antifungal, Arachidonic acid, Biofilms, Clotrimazole, Eicosanoids, Fatty acid, Metabolic activity, Phenotypic characteristics, Phospholipids, Planktonic cells.

Opsomming

Candida albicans en *C. dubliniensis* is twee nabyverwante patogeniese gisspesies, wat verskeie fenotipiese eienskappe in gemeen het. Dit maak dit moeilik om tussen die twee spesies te onderskei, veral in kliniese monsters. As gevolg hiervan is identifikasietegnieke, gebaseer op fenotipiese eienskappe, ontwikkel. In hierdie studie is daar van die tegnieke en virulensiefaktore gebruik gemaak om stamme wat aan die spesies behoort, te karakteriseer en sodoende fenotipies verskillende stamme te selekteer vir gebruik in verdere studies. Dit was gedoen om te bepaal of die effek van arachidoonsuur (20:4) op hierdie stamme dieselfde is, alhoewel hulle fenotipies verskil. *Candida albicans* en *C. dubliniensis* is in staat om biofilms, wat 'n belangrike rol speel tydens infeksies, te vorm. Tydens *C. albicans* infeksies word 20:4, 'n lang-ketting poli-onversadigde vetsuur uit die fosfolipiede van die geïnfekteerde gasheer se sel membraan vrygestel. Dit dien dan as koolstofbron en as voorloper vir eikosanoïedproduksie. In literatuur is daar teenstrydige resultate in verband met die effek van 20:4 op die groei en morfogenese in *C. albicans*. Boonop is die effek van 20:4 op die groei en morfologie van *C. dubliniensis* nog onbekend. Deur mikroskopiese ondersoek en ensiemaktiwiteitstoetse is bevind dat 1 mM 20:4 min tot geen effek op die groei en metaboliese aktiwiteit van planktoniese selle en biofilms asook op die morfologie en lewensvatbaarheid van selle in die biofilm het nie. Die opname van lang-ketting poli-onversadigde vetsure deur giste is noodsaaklik vir gebruik as metaboliese energiebronne, sellulêre boustene en vir die produksie van seinmolekules. Daar is egter geen bepaalde studies wat die opname en sellulêre metabolisme van 20:4 deur die patogeniese giste aandui nie. Die opname en inkorporasie van 20:4 deur planktoniese selle en biofilms van die geselekteerde *C. albicans* en *C. dubliniensis* stamme is dus bestudeer. Dit is gedoen deur die oorblywende en sellulêre lipiede van planktoniese selle en biofilms, gegroei in die teenwoordigheid en afwesigheid van 20:4, te analiseer m.b.v. gaschromatografie en gaschromatografie-massaspektrometrie. Die resultate het variasie in die opname en inkorporasie van 20:4 in die veskillende lipied fraksies tussen stamme van planktoniese selle en biofilms aangedui. Daarby is eikosanoïede, geproduseer deur biofilms in die teenwoordigheid van 20:4, ontrek en is dit bevind dat al die stamme in

staat is om 3-hidroksievetsure vanaf 20:4 te produseer. Arachidoonsuur word in die fosfolipiede van giste ingebou en beïnvloed daardeur die versadiging in die selmembrane. Daar is aangedui dat die effektiwiteit van antifungale kan afhang van die vlak van onversadiging en die ergosterolinhoud van die membrane. Dus is die effek van 20:4 op die selmembrane en die vatbaarheid van *C. albicans* en *C. dubliniensis* biofilms teenoor amfoterisien B en klotrimazool bepaal. Dit is gedoen deur konfokale laserskandeermikroskopie, bepaling van die mitochondriale metabolisme, onversadiging van die fosfolipiedfraksies en die ergosterolinhoud in die membrane van biofilms gegroei in die teenwoordigheid en afwesigheid van 20:4. Die resultate het aangedui dat 20:4 die onversadiging van die fosfolipiede en die ergosterolinhoud van die tipestamme van *C. albicans* en *C. dubliniensis* beïnvloed, asook lei tot ’n verhoging in die vatbaarheid vir die antifungale. Voor-behandeling van biofilms met lang-ketting poli-onversadigde vetsure mag dus lei tot die vermindering in die dosis antifungaal benodig om die biofilms te inhibeer.

Sleutelwoorde:
Amfoterisien B, Antifungaal, Arachidoonsuur, Biofilms, Eikosanoïede, Fenotipiese eienskappe, Fosfolipiede, Klotrimazool, Metaboliese aktiwiteit, Planktoniese selle, Vetsuur.