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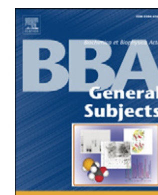

# Polyunsaturated fatty acids cause apoptosis in *C. albicans* and *C. dubliniensis* biofilms

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ABSTRACT

*Background:* Polyunsaturated fatty acids (PUFAs) have antifungal properties, but the mode by which they induce their action is not always clear. The aim of the study was to investigate apoptosis as a mode of action of antifungal PUFAs (stearidonic acid, eicosapentaenoic acid and docosapentaenoic acid) which are inhibitory towards biofilm formation of *C. albicans* and *C. dubliniensis*.
*Methods:* *Candida* biofilms were grown in the absence or presence of 1 mM PUFAs (linoleic acid, stearidonic acid, eicosapentaenoic acid, docosapentaenoic acid) for 48 h at 37 °C. The effect of these PUFAs on the membrane fatty acid profile and unsaturation index, oxidative stress, mitochondrial transmembrane potential and apoptosis was evaluated.
*Results:* When biofilms of *C. albicans* and *C. dubliniensis* were exposed to certain PUFAs there was an increase in unsaturation index of the cellular membranes and accumulation of intracellular reactive oxygen species (ROS). This resulted in apoptosis, evidenced by reduced mitochondrial membrane potential and nuclear condensation and fragmentation. The most effective PUFA was stearidonic acid.
*Conclusions:* The resultant cell death of both *C. albicans* and *C. dubliniensis* is due to apoptosis.
*General significance:* Due to the increase in drug resistance, alternative antifungal drugs are needed. A group of natural antifungal compounds is PUFAs. However, understanding their mechanisms of action is important for further use and development of these compounds as antifungal drugs. This paper provides insight into a possible mode of action of antifungal PUFAs.

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## 1. Introduction

In an earlier study [1] it was found that certain marine polyunsaturated fatty acids (PUFAs) [i.e. stearidonic acid (C18:4 n-3), eicosapentaenoic acid (C20:5 n-3) and docosapentaenoic acid (C22:5 n-3)] cause inhibition of *Candida albicans* and *Candida dubliniensis* biofilm biomass production as well as metabolic activity. The authors speculated that these effects may be due to oxidative stress, which could lead to apoptosis.

Apoptosis is characterized by a set of distinct morphological markers and yeast cells show typical apoptotic markers such as the externalization of phosphatidylserine, DNA fragmentation as well as chromatin fragmentation and condensation. Furthermore, the cytochrome c release from the mitochondria [2], reduced mitochondrial membrane potential [3] and formation of membrane enclosed cell fragments, called apoptotic bodies, all mark programmed death of yeast cells [4]. Madeo and co-workers [5] first discovered the apoptotic pathway similar to those in multicellular organisms, in the yeast *Saccharomyces cerevisiae*. Since then several endogenous and exogenous stimuli have been shown to be responsible for the induction of apoptosis in this yeast. Some of these stimuli include mutations or deletions of specific genes [5] and exogenous stimuli such as acetic acid and hydrogen peroxide [6–8]. Research has also shown the existence of apoptotic pathways in the human pathogen *Candida albicans*. In this yeast, antimicrobial peptides such as melittin [9], psacotheasin [10], pleurocidin [11], papiliocin [12] and arenicin-1 [13] were found to be antifungal through the induction of apoptosis. These peptides were reported to induce oxidative stress through increased production of reactive oxygen species (ROS) and lipid peroxidation of mitochondrial membranes. Accumulation of ROS has long been proposed to be a key event in the apoptotic pathway [14].

The toxicity of fatty acids is dose dependent and relates to chain length and unsaturation, especially with PUFAs, causing mammalian cell death through apoptosis [15]. In the recent work by Ferreira and co-workers [16], it was reported that supplementation of media with PUFAs could induce the apoptotic pathway in *S. cerevisiae*.

The aim of the study was therefore to investigate whether the observed antifungal activity of marine PUFAs, as reported by Thibane and co-workers [1], is through the induction of apoptosis in *C. albicans* and *C. dubliniensis* biofilms.

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## 2. Materials and methods

### 2.1. Strains used

*Candida albicans* CBS 562 T and *Candida dubliniensis* NRRL Y-17841 T were used in this study and were maintained on yeast malt extract (YM) agar plates (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 16 g/L agar) at room temperature. The strains were also stored on agar slants at 4 °C.

### 2.2. Biofilm formation

Cells of *C. albicans* and *C. dubliniensis* were grown separately on YM agar plates and incubated at 30 °C for 24 h. After incubation, a loop-full of the cells was inoculated into 20 mL of yeast nitrogen base (YNB) glucose medium (10 g/L glucose, 6.7 g/L YNB) and incubated at 30 °C for 48 h. Cells were washed twice with sterile phosphate buffered saline (PBS) by centrifugation for 5 min at 4000 ×*g* with a Heraeus® Megafuge® 1.0R Centrifuge and diluted in filter sterilized RPMI-1640 medium (Sigma Aldrich, USA) to a standardized cell concentration of $1 \times 10^6$ cells/mL. Biofilms were allowed to form by first incubating the cells at 37 °C for 1 h, to allow adherence of cells to the surface [17]. Non-adherent cells were removed by washing twice with sterile PBS. Different PUFAs [linoleic acid (C18:2 n-6), C18:4 n-3, C20:5 n-3 and C22:5 n-3] with a final concentration of 1 mM, as used in Thibane and co-workers [1], were added after 1 hour incubation and mature biofilms were formed at 37 °C for an additional 47 h. Ethanol was used as a control.

### 2.3. Phospholipid fatty acid analyses

Biofilms, formed in Petri dishes and supplemented with PUFAs, were washed twice with sterile PBS, scraped off using a cell scraper and resuspended in sterile PBS. Total lipids of the biofilms were extracted with chloroform:methanol (2:1) [18]. The total lipids were separated into different fractions using solid phase 0.50 g Si extraction columns (Separations, SA) according to the method of Bossio and Scow [19]. Briefly, columns were conditioned with 2 mL chloroform followed by transfer of the lipid samples, resuspended in 300 μL chloroform, to the columns. The neutral lipids were eluted with 5 mL chloroform, followed by the glycolipids with 10 mL acetone and finally the phospholipids with 5 mL methanol. The eluates were collected and dried under $N_2$ at 32 °C. Fatty acid methyl esters (FAMEs) of the phospholipid fractions were prepared using methanol-$BF_3$ [20] and quantified using a Varian 430-GC gas chromatograph, with a fused silica capillary column (Chrompack CPSIL 88, 100 m length, 0.25 mm ID, 0.2 μm film thickness). The column temperature was 40–230 °C (hold 2 min; 4 °C/min; hold 10 min). Fatty acid methyl esters in hexane (1 μL) were injected into the column using a Varian CP-8400 Autosampler with a split ratio of 100:1. The injection port and detector temperatures were maintained at 250 °C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the makeup gas. Galaxy Chromatography Data System software recorded the chromatograms. Fatty acid methyl ester samples were identified by comparing the relative retention times of FAMEs peaks from samples with those of standards. Fatty acids were expressed as the relative percentage of each individual fatty acid to the total of all fatty acids present in the samples. The unsaturation index was calculated using the following equation: Unsaturation Index = 1 × [% monoenoic fatty acids] + 2 × [% dienoic fatty acids] + 3 × [% trienoic fatty acids] + 4 × [% tetraenoic fatty acids] + 5 × [% pentaenoic fatty acids]. This experiment was performed in duplicate on different occasions.

### 2.4. Measurement of reactive oxygen species (ROS)

Accumulation of intracellular ROS production was measured using the fluorescent dye, 2',7-dichlorofluorescin diacetate (DCFHDA) (Sigma Aldrich, USA). Briefly, biofilms were prepared as described above in black 96-well microtiter plates (Corning Incorporated, Costar®, USA) by incubating plates at 37 °C for 24 h. Biofilms were washed twice with sterile PBS to remove non-adherent cells and 100 μL of filter sterilized RPMI-1640 medium containing 1 mM of the PUFAs were added. An ethanol control was included. A volume of 10 μM DCFHDA was simultaneously added with the PUFAs and the plate was incubated at 37 °C for an additional 12 h [21]. Fluorescence was measured after incubation using a SpectraMax M2 plate reader (Molecular Devices, USA) at an excitation/emission wavelength of 485/535 nm, respectively.

### 2.5. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential of biofilms prepared in black 96-well microtiter plates, and supplemented with PUFAs as described above, was measured. After incubation, wells were washed twice with sterile PBS and the plates assayed according to the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman chemicals, USA) using a SpectraMax M2 plate reader. The lipophilic cationic dye, 5,5′,6,6′-terachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), selectively enters the mitochondria. In healthy cells, with high mitochondrial transmembrane potential ($\Delta\Psi$m), JC-1 forms J-aggregates with an intense red fluorescence at excitation/emission wavelength of 540/570 nm, respectively. In apoptotic or unhealthy cells, with low $\Delta\Psi$m, JC-1 remains in the monomeric form which fluoresces green at excitation/emission wavelength of 485/535 nm, respectively [22]. Mitochondrial membrane potential was presented as the ratio of the J-aggregates (healthy cells) and monomeric forms (apoptotic cells).

### 2.6. DNA fragmentation (TUNEL) assay

For the analyses of DNA strand breaks, biofilms were prepared in Petri dishes and supplemented with PUFAs as described above. Cells were washed twice with PBS and fixed in 3.6% formaldehyde. Fixed cells were washed twice with PBS and protoplasted as described and permeabilized in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. DNA strands were labeled with an *In Situ* Cell Death Detection Fluorescein Kit (Roche Applied Science, Germany) at 37 °C for 1 h [23]. Cells were analyzed on the FACSCalibur Flowcytometer using CellQuest Pro software. At least 75 000 cells were counted per analyses. Instrument-settings for standard human platelets were used. Hydrogen peroxide (10 mM) was used as positive control.

**Table 1**
Phospholipid fatty acid profiles of biofilms of *Candida albicans* following supplementation with C18:2 n-6, C18:4 n-3, C20:5 n-3 and C22:5 n-3. Values represent mean of duplicate experiments with the range in brackets.

| Fatty acid | Relative % | | | | |
|---|---|---|---|---|---|
| | Control | C18:2 n-6 | C18:4 n-3 | C20:5 n-3 | C22:5 n-3 |
| **C16:0** | 34.8 (7.2) | 38.8 (14.2) | 28.9 (4.3) | 22.8 (3.1) | 29.2 (5.1) |
| **C16:1 n-9** | 5.3 (3.5) | 4.2 (2.1) | 1.7 (0.6) | 1.3 (0.6) | 3.0 (1.5) |
| **C18:0** | 23.5 (4.0) | 18.1 (1.6) | 20.4 (5.0) | 13.6 (1.8) | 21.1 (5.6) |
| **C18:1 n-9** | 27.0 (0.3) | 17.5 (4.6) | 17.5 (2.5) | 14.6 (3.2) | 31.4 (4.3) |
| **C18:2 n-6** | 8.6 (1.4) | 22.6 (8.2) | 8.5 (2.2) | 7.6 (1.4) | 9.7 (0.2) |
| **C18:3 n-3** | 0.9 (0.4) | 1.1 (0.6) | 0.7 (0.3) | 0.6 (0.3) | 1.1 (0.6) |
| **C18:4 n-3** | – | – | **21.9 (15.4)** | – | – |
| **C20:0** | 0.6 (0.3) | 0.4 (0.2) | 0.6 (0.3) | 0.4 (0.2) | 0.3 (0) |
| **C20:1 n-9** | – | – | – | – | 0.1 (0.1) |
| **C20:2 n-6** | – | 0.1 (0) | – | – | 0.1 (0) |
| **C20:3 n-3** | – | – | – | – | – |
| **C20:4 n-6** | – | – | – | – | 0.2 (0.1) |
| **C20:5 n-3** | 0.3 (0.2) | 0.3 (0.1) | 0.5 (0.2) | **40.2 (4.3)** | 2.6 (1.3) |
| **C22:5 n-3** | – | – | – | – | 3.0 (2.8) |

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