


Antibiofilm Activity of Essential Fatty Acids Against *Candida albicans* from Vulvovaginal Candidiasis and Bloodstream Infections

Shuai Wang¹, Peng Wang¹, Jun Liu¹, Chunxia Yang¹, Qiangyi Wang², Mingze Su³, Ming Wei¹ , Li Gu¹

¹Department of Infectious Diseases and Clinical Microbiology, Beijing Institute of Respiratory Medicine and Beijing Chao-Yang Hospital, Capital Medical University, Beijing, People's Republic of China; ²Department of Clinical Laboratory, Beijing Hospital, Chinese Academy of Medical Sciences, Beijing, People's Republic of China; ³Department of Clinical Laboratory, Beijing Tongren Hospital, Capital Medical University, Beijing, People's Republic of China

Correspondence: Ming Wei; Li Gu, Department of Infectious Diseases and Clinical Microbiology, Beijing Institute of Respiratory Medicine and Beijing Chao-Yang Hospital, Capital Medical University, 8 Gongren Tiyuchang Nanlu, Chaoyang District, Beijing, 100020, People's Republic of China, Tel +86-10-85231513, Email weiming@bjmu.edu.cn; guli2013227@foxmail.com

Purpose: The biofilm formation of *Candida albicans* is an important virulence factor as it can increase tolerance to conventional antifungal drugs and the host immune system. The study aimed to assess the effect of essential fatty acids (EFAs) against biofilm formation and mature biofilms of *C. albicans* strains, which were isolated from vulvovaginal candidiasis and candidemia.

Methods: The biofilm formation ability of *C. albicans* and antifungal activities of fluconazole were determined. Additionally, the effects of six EFAs [α -linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LOA), γ -linolenic acid (GLA), and arachidonic acid (AA)] against *C. albicans* under planktonic and biofilm conditions were evaluated.

Results: 94.1% of *C. albicans* exhibited biofilm formation capacity, and 98.5% of *C. albicans* were susceptible to fluconazole. The biofilms of *C. albicans* were highly resistant to fluconazole with minimum biofilm eradication concentration values ≥ 64 $\mu\text{g/mL}$. The EFAs attenuated biofilm formation in a dose-dependent manner, and GLA displayed a remarkable inhibitory activity against biofilm formation of *C. albicans*. In addition, EPA, DHA, and GLA at 0.1 mM could inhibit the biofilm formation of *C. albicans* without affecting the planktonic growth rate. Notably, EPA and AA at 1 mM had both inhibitory and eradication activities on *C. albicans* biofilms.

Conclusion: This is the first study to directly compare different EFAs for their capacity to affect *C. albicans* biofilm formation as well as biofilm eradication. These results suggest EPA and AA could serve as potential new antifungal agents for the treatment of clinical infections caused by *C. albicans* biofilms.

Keywords: *Candida albicans*, polyunsaturated fatty acids, biofilm, inhibition, eradication

Introduction

Candida species are the most common human fungal pathogens.^{1,2} Although clinical infections caused by non-*albicans* *Candida* spp. (eg, *C. tropicalis*, *C. glabrata*, and *C. parapsilosis*) have increased in recent years, *C. albicans* remains the most predominant *Candida* spp. causing infectious diseases in both adult and pediatric populations.³ Most recurrent and severe infections by *C. albicans* are associated with the formation of biofilms on the surface of biological or artificial surfaces.⁴ *Candida* biofilms are complex communities of yeast and filamentous cells surrounded by an extracellular matrix.⁵ The biofilm formation of *C. albicans* is an essential virulence factor because it can increase tolerance to conventional antifungal drugs and the host immune system.⁶

Candida spp. can invade various tissues and organs of the human body and are responsible for superficial (mucosal and cutaneous) and systemic infection. Vulvovaginal candidiasis (VVC) is a common infection of the vaginal mucosa caused by *Candida* spp.⁷ It is estimated that 70%-75% of women experience VVC at least once in their lifetime, with 40%-50% of these women experiencing recurrent VVC (RVVC).⁸ The ability of *Candida* spp. to form biofilms is crucial

in recurrent VVC.⁹ *Candida* bloodstream infection (candidemia) is a severe systemic infection, with a crude mortality rate of 25%-50%.^{10,11} Recent studies have shown that the biofilm formation of *Candida* spp. is associated with higher mortality in patients with candidemia.¹²⁻¹⁵

Due to the increasing occurrence of *Candida* biofilm-related infections and their immense impact on the health care system, there is an urgent need to find novel efficient antifungal agents. Fatty acids, which are promising to be considered as the next generation of antimicrobial agents to treat bacterial infections,¹⁶ are also found to exhibit significant inhibitory effects against *Candida* spp. and have recently been considered potential alternative antifungal agents.¹⁷⁻²² Nevertheless, few published studies have investigated the activities of essential fatty acids (EFAs) on biofilms in the formation and mature stages of clinical *C. albicans* isolates. EFAs, which are types of polyunsaturated fatty acids (PUFAs), cannot be synthesized by the human body and must be obtained in the diet. It has been suggested that some PUFAs act as antibiofilm agents through functioning as molecular signals, as well as influencing PUFA metabolism and other metabolic activities.^{17,19}

The EFAs are classified into two series, the omega-3 (ω -3) and the omega-6 (ω -6).²³ The ω -3 series is derived from α -linolenic acid (ALA, 18:3). The ω -6 series is derived from linoleic acid (LOA, 18:2). ALA is metabolized to eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6); and LOA is metabolized to γ -linolenic acid (GLA; 18:3) and arachidonic acid (AA, 20:4) (Figure 1). This study aimed to investigate the effect of six EFAs against biofilm formation and mature biofilms of *C. albicans* strains, which were isolated from VVC and candidemia.

Materials and Methods

Chemicals

The EFAs, namely α -linolenic acid (ALA; 18:3 ω -3), eicosapentaenoic acid (EPA; 20:5 ω -3), docosahexaenoic acid (DHA; 22:6 ω -3), linoleic acid (LOA; 18:2 ω -6), γ -linolenic acid (GLA; 18:3 ω -6), and arachidonic acid (AA; 20:4 ω -6) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluconazole was purchased from Solarbio (Beijing, China). Dimethyl sulfoxide (DMSO) was used as a solvent to dissolve the EFAs and fluconazole. Stock solutions were kept at

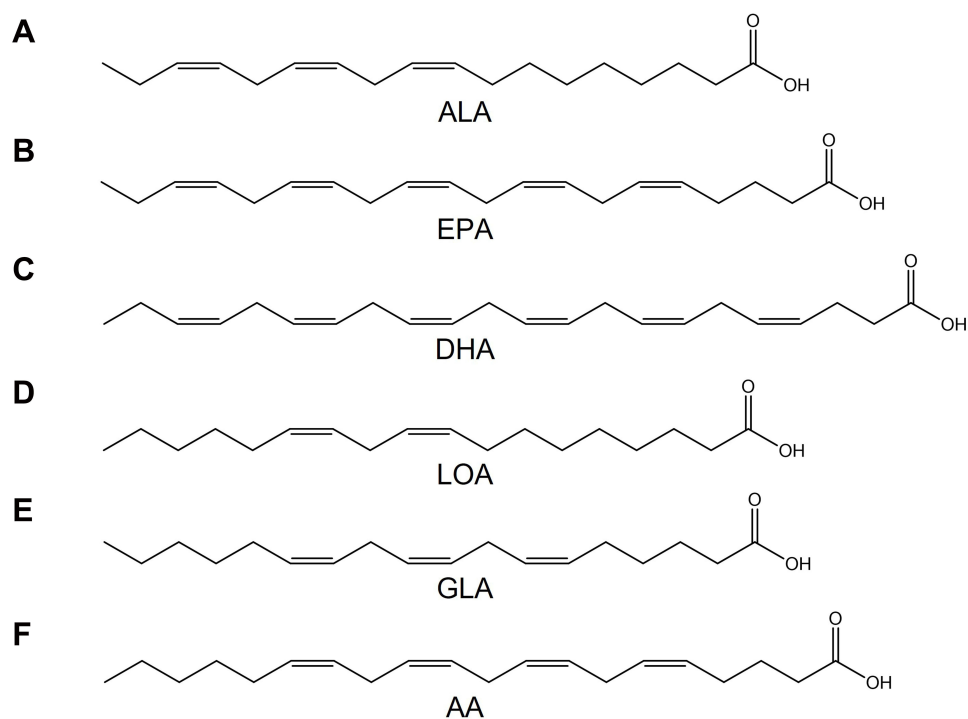


Figure 1 Chemical structure of EFAs. (A) ALA, α -linolenic acid. (B) EPA, eicosapentaenoic acid. (C) DHA, docosahexaenoic acid. (D) LOA, linoleic acid. (E) GLA, γ -linolenic acid. (F) AA, arachidonic acid.

–20°C for a maximum of 4 weeks and were further diluted in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) before use. DMSO at <0.1% did not affect microbial growth or biofilm formation.

Strains and Culture Conditions

A total of 135 clinical non-duplicate *C. albicans* isolates were collected from the Department of Infectious Diseases and Clinical Microbiology, Beijing Chao-Yang Hospital, Capital Medical University (Beijing, China) [vaginal isolates (n=70) from patients diagnosed with VVC and blood isolates (n=65) from patients diagnosed with candidemia]. *C. albicans* ATCC 90028 was used as the reference control. All *C. albicans* isolates were identified using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (VITEK-MS; bioMérieux, France; IVD version 3.0), and further confirmed by PCR sequencing of the ITS region of rDNA. *C. albicans* strains were routinely refreshed from the frozen stocks at –20°C and inoculated at least twice onto Sabouraud Dextrose Agar (SDA) at 35°C for 24 h before all experiments.

Determination of Minimum Inhibitory Concentration of Fluconazole

To determine the minimum inhibitory concentration (MIC) of fluconazole against *C. albicans* strains, a broth micro-dilution method was used as described by CLSI M27-A4.²⁴ The MIC was defined as the lowest concentration at which there is a 50% decrease in the growth of planktonic *C. albicans*. Briefly, the initial concentration of the *C. albicans* suspension was adjusted to 1×10^3 CFU/mL in RPMI 1640 medium. The final concentrations of fluconazole were 0.125–64 µg/mL. The MICs were determined spectrophotometrically at OD_{630 nm} following a 24h incubation period at 35°C. *C. albicans* ATCC 90028 was used as a quality control strain. All isolates were measured in triplicate.

Biofilm Formation by Crystal Violet Method

To measure the ability of *C. albicans* to form a biofilm, the crystal violet assay in sterile 96-well round-bottomed polystyrene plates (Corning, NY, USA), was performed as described previously.²⁵ Briefly, *C. albicans* cells were harvested from overnight cultures and inoculated into RPMI 1640 medium at a dilution of 1:100. Then, 200 µL of suspension (1×10^5 CFU/mL) was added to each well of the microtiter plate and incubated at 35°C in an atmosphere of 5% CO₂ for 24 h.

To determine the biofilm mass, the wells were washed three times with pH 7.2 phosphate-buffered saline (PBS) to remove planktonic cells. The remaining attached cells were fixed with 200 µL methanol for 15 min, stained with 200 µL crystal violet (0.2%) for 20 min, and washed with distilled water to remove the excess crystal violet. Then, the bound crystal violet was extracted with 200 µL of 33% acetic acid. The OD values were measured at 630 nm using a Multiskan EX microplate photometer (Thermo Fisher Scientific, Waltham, MA, United States). Experiments were independently repeated three times. Wells with RPMI 1640 medium were used as negative controls. The OD cut-off value (OD_c) for biofilm formation was defined as three standard deviations above the mean OD value of the negative control.

The isolates were categorized as strong biofilm producers (OD_{630 nm} > 4×OD_c), moderate biofilm producers (2×OD_c < OD_{630 nm} ≤ 4×OD_c), weak biofilm producers (OD_c < OD_{630 nm} ≤ 2×OD_c), and non-biofilm producers (OD_{630 nm} ≤ OD_c).²⁶

Inhibition of Biofilm Formation

To detect the effects of fluconazole or EFAs treatment on biofilm formation of *C. albicans*, the cell suspensions were co-cultured with different concentrations of fluconazole (0.125–256 µg/mL) or EFAs (0.01, 0.1, and 1 mM) in microtiter plates. RPMI 1640 medium without fluconazole or EFAs was added to the wells as the control for biofilm formation. Then, the plates were incubated at 35°C in 5% CO₂ for 24 h. Blank solvent control (RPMI 1640 medium supplemented with 0.1% DMSO) and blank medium control (RPMI 1640 medium) were also performed. The biofilm mass was detected using the crystal violet method as described above. EFAs at ≤ 1 mM did not affect the biofilm detection.

The minimum biofilm inhibitory concentration (MBIC) was defined as the minimum concentration of antifungal agents, leading to a 50% reduction of biofilm formation compared to the control group. The biofilm inhibition rate (%) was calculated according to the formula:²⁷

$$\text{Inhibition (\%)} = (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100$$

Eradication of Mature Biofilm

To measure the biofilm eradication ability of fluconazole or EFAs against *C. albicans*, the mature biofilms of *C. albicans* were pre-formed by adding only cell suspensions and culturing for 24h. After incubation, the non-adherent cells were removed by washing with PBS twice. Then, RPMI 1640 medium containing different concentrations of fluconazole (0.125–256 µg/mL) or EFAs (0.01, 0.1, and 1 mM) were added to the wells. The mature biofilm treated with fresh RPMI 1640 medium served as biofilm control. The microtiter plates were incubated at 35°C for an additional 24 h. Blank solvent control (RPMI 1640 medium supplemented with 0.1% DMSO) and blank medium control (RPMI 1640 medium) were also performed. The biofilm mass was detected using the crystal violet method as described above. EFAs at ≤ 1 mM did not affect the biofilm detection.

The minimum biofilm eradication concentration (MBEC) was defined as the minimum concentration of antifungal agents that eradicated mature biofilm by 50% compared to the control group. The biofilm eradication rate (%) was calculated according to the formula:²⁷

$$\text{Eradication (\%)} = (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / \text{OD}_{\text{control}} \times 100$$

Evaluation of the Effect of Essential Fatty Acids on Fluconazole Susceptibility

To evaluate the effect of EFAs on fluconazole susceptibility, the MICs, MBICs, and MBECs of fluconazole (0.125–256 µg/mL) combined with different EFAs (0.1 mM) on *C. albicans* strains were tested as described above. Growth and blank controls were also included. The MICs were determined spectrophotometrically at OD_{630 nm}. The MBICs and MBECs were detected using the crystal violet method.

Growth Curves of *C. albicans*

Growth curves for *C. albicans* strains were established in the absence and presence of EFAs (0.01, 0.1, and 1 mM). The assay was performed using the HB&L system (Alifax, Polverara, Italy) as previously described.²⁸ Briefly, *C. albicans* cells were inoculated into 2 mL of RPMI 1640 medium (1:100) with an initial concentration of 1×10⁵ CFU/mL. The inoculated culture was grown at 35 °C under gentle agitation, and the McF was determined every hour until 14 h.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 statistical software (IBM, Armonk, NY, USA). Data were expressed as means ± standard deviation (SD) of at least three independent experiments. The chi-squared test was used to compare qualitative variables. Statistical differences between biofilms biomass in treated and untreated groups were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett-*t* test. A comparison of the MIC, MBIC, and MBEC values of fluconazole alone and combined EFAs was performed using the Wilcoxon Signed Rank Test. A *P*-value < 0.05 was considered statistically significant.

Results

Biofilm Formation Ability of *C. albicans*

Of the total 135 *C. albicans* isolates studied, 70 were from women diagnosed with VVC, and 65 were obtained from the blood cultures of patients with candidemia. The biofilm formation ability of *C. albicans* was determined using the crystal violet method (Figure 2). A total of 94.1% (127/135) of *C. albicans* isolates exhibited biofilm formation capacity, out of which 65.2%, 20.0%, and 8.9% showed strong, moderate, and weak biofilm formation capacity, respectively. 94.3% (66/70) and 93.8% (61/65) were biofilm producers among VVC-derived strains and candidemia-derived strains, respectively.

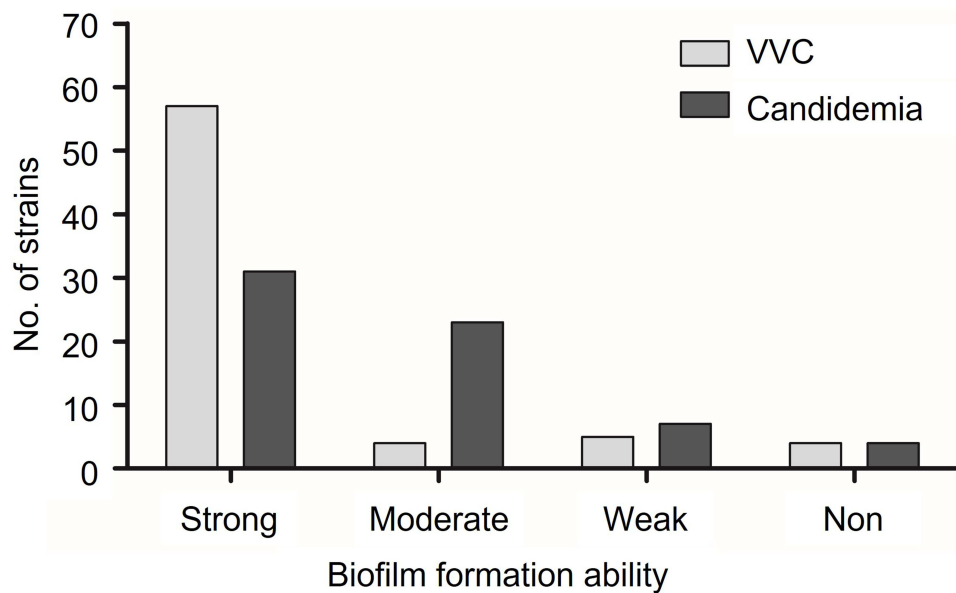


Figure 2 The biofilm formation ability of *C. albicans* strains from vulvovaginal candidiasis (VVC) and candidemia.

Moreover, strong biofilm producers were significantly more frequent among VVC-derived strains (57/70, 81.4%) than among candidemia-derived strains (31/65, 47.7%) ($\chi^2 = 16.903$, $P < 0.05$).

Minimum Inhibitory Concentration of Fluconazole for Planktonic *C. albicans*

As shown in Figure 3, the minimum inhibitory concentration (MIC) values of fluconazole for 135 isolates of *C. albicans* ranged from ≤ 0.125 to 8 $\mu\text{g}/\text{mL}$. The distribution of the MICs was similar between the VVC-derived and candidemia-derived strains. Most strains obtained MIC values that were ≤ 0.125 $\mu\text{g}/\text{mL}$ (118/135, 87.4%). According to the CLSI M27-A4 guideline,²⁴ 98.5% (133/135) of *C. albicans* were susceptible to fluconazole (MIC ≤ 2 $\mu\text{g}/\text{mL}$). Only one *C. albicans* strain (Y65) isolated from VVC appeared resistant to fluconazole with a MIC = 8 $\mu\text{g}/\text{mL}$.

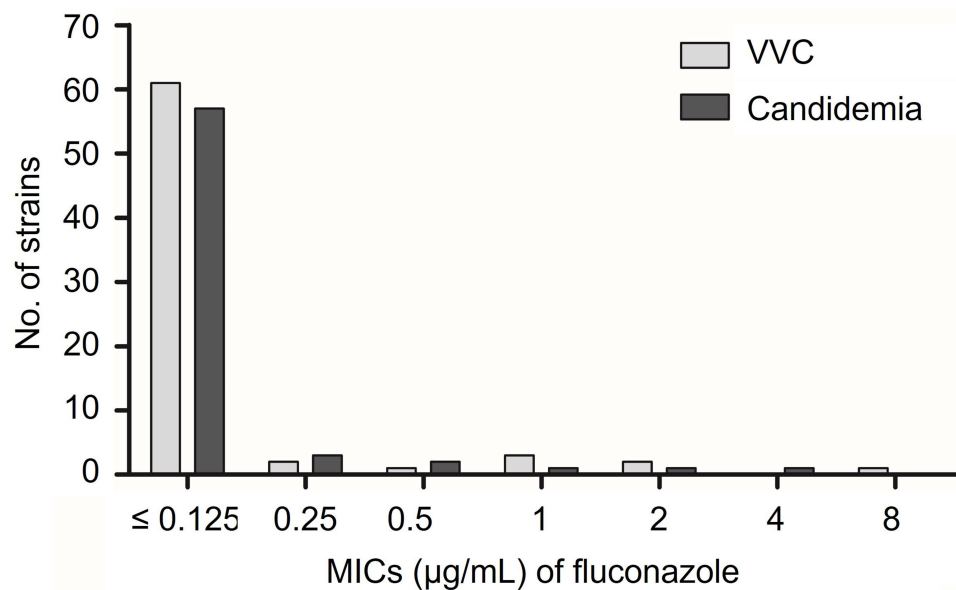


Figure 3 Distribution of the minimum inhibitory concentrations (MICs) of fluconazole among *C. albicans* strains from vulvovaginal candidiasis (VVC) and candidemia.

Table 1 The MICs, MBICs, and MBECs of Fluconazole Against *C. albicans* Strains

<i>C. albicans</i> Strains	Source	MIC ($\mu\text{g/mL}$)	MBIC ($\mu\text{g/mL}$)	MBEC ($\mu\text{g/mL}$)
Y02	Vaginal specimen	0.25	1	128
Y20	Vaginal specimen	2	2	128
Y26	Vaginal specimen	1	4	256
Y37	Vaginal specimen	0.25	1	256
Y38	Vaginal specimen	0.5	0.5	> 256
Y65	Vaginal specimen	8	8	> 256
Y70	Vaginal specimen	1	1	> 256
X06	Blood culture	0.5	0.5	128
X09	Blood culture	0.25	0.25	64
X52	Blood culture	1	1	> 256
X54	Blood culture	2	2	256
X55	Blood culture	0.25	0.25	256

Abbreviations: MIC, minimum inhibitory concentration; MBIC, minimum biofilm inhibitory concentration; MBEC, minimum biofilm eradication concentration.

Antibiofilm Effect of Fluconazole

The minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) values of fluconazole were tested against 12 clinical strains with a strong biofilm-forming ability and MIC > 0.125 $\mu\text{g/mL}$ (Table 1). The MBIC values, which ranged from 0.25 to 8 $\mu\text{g/mL}$ for biofilms, were similar to the corresponding strains' MIC values for planktonic cells. However, the MBEC values increased drastically compared with the MIC values, ranging 64- to 1024- fold. The biofilms of *C. albicans* appeared to be highly resistant to fluconazole with MBEC values above 64 $\mu\text{g/mL}$.

Inhibitory Effect of Essential Fatty Acids on Biofilm Formation

To determine whether EFAs inhibited the biofilm formation of 12 strains of *C. albicans*, the biofilm biomass of EFAs treated and control groups were compared. As shown in Figure 4, after 24 h of treatment with EFAs at a concentration of 0.01 mM, none of the EFAs had inhibitory effects on biofilm formation; whereas in the presence of the highest

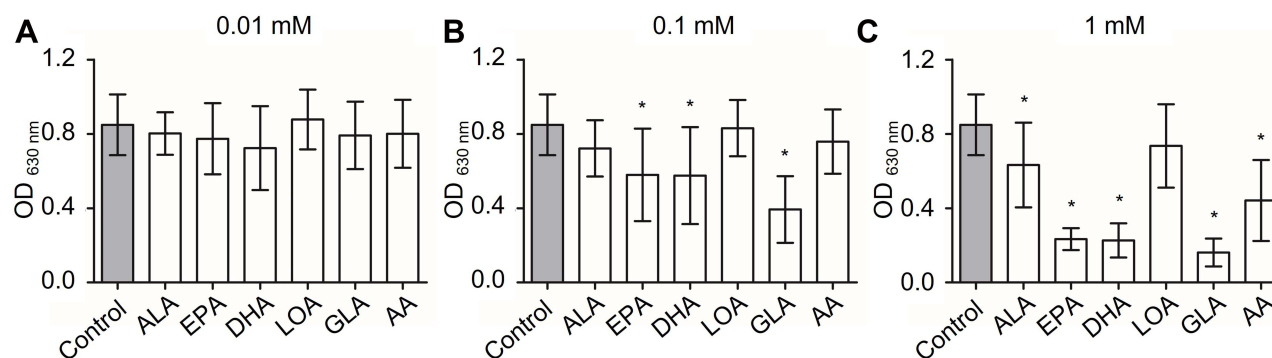


Figure 4 Inhibitory effect of essential fatty acids (EFAs) on biofilm formation of *C. albicans*. (A) 0.01 mM EFAs. (B) 0.1 mM EFAs. (C) 1 mM EFAs. The values shown are means \pm standard deviations. An asterisk denotes a statistically significant difference compared to the untreated control ($P < 0.05$).

concentration (1 mM) tested, except for LOA, other EFAs had inhibitory effects on biofilm formation ($P < 0.05$), and the EFAs attenuated biofilm formation in a dose-dependent manner. In addition, the most pronounced inhibitory effect on biofilm formation was observed for GLA, with the biofilm inhibition rates (%) at 0.01 mM, 0.1 mM, and 1 mM being 13.35%, 54.36%, and 80.99%, respectively (Table S1).

Eradication Effect of Essential Fatty Acids on Mature Biofilm

The eradication biofilm activity of EFAs was evaluated against preformed 24 h biofilms of 12 *C. albicans* isolates (Figure 5). At concentrations of 0.01 mM and 0.1 mM, there were no significant effects on the mature biofilms of *C. albicans* compared with the control group ($P > 0.05$). Nevertheless, at the increased concentration (1 mM), the biofilm of *C. albicans* was significantly eradicated by EPA and AA ($P < 0.05$), and the biofilm eradication rates (%) were 29.95% and 24.14%, respectively (Table S2). Notably, EPA and AA at 1 mM had both eradication and inhibitory activities on the biofilms of *C. albicans* (Figures 4 and 5).

Effects of EFAs on Fluconazole Susceptibility

To evaluate the impact of EFAs on fluconazole susceptibility, the MICs, MBICs, and MBECs of fluconazole combined with different EFAs (0.1 mM) on clinical *C. albicans* strains were determined. It was observed that under the planktonic condition the MIC values were increased after fluconazole combined with EFAs ($P < 0.05$) (Table 2). Under the biofilm condition no significant differences ($P > 0.05$) were found between the combination treatment and fluconazole alone treatment (Tables 3 and 4).

Effects of EFAs on *C. albicans* Growth

To further understand whether EFAs affect the growth rate of *C. albicans* and thus play an antibiofilm role, the growth curves of four *C. albicans* strains (Y26, Y38, Y65, and X52) were measured. These four strains were relatively sensitive to EFAs under biofilm conditions. As shown in Figure 6, None of the EFAs suppressed the growth of *C. albicans* at the low concentrations (0.01 mM and 0.1 mM), while EPA, DHA, GLA, and AA at 1 mM had inhibitory effects on growth.

Discussion

C. albicans has been associated with many human infections and causes considerable morbidity and mortality.²⁹ One of the most potent pathogenic traits of *C. albicans* is its capacity to form a biofilm. Biofilms allow for enhanced adhesion to body surfaces, protection from environmental stressors and the host immune system, and often result in increased tolerance to antifungal agents.⁶ According to these properties, the biofilm formation of *C. albicans* is an important vital determinant during candidiasis.⁶ As traditional antifungal drugs do not work well against the biofilms, the present study intended to search for new antifungal agents to control *C. albicans* biofilms.

In this study, a total of 135 clinical isolates of *C. albicans* (vaginal isolates, n=70; blood isolates, n=65) were determined for the biofilm formation ability. The vast majority of *C. albicans* showed biofilm formation ability (94.1%,

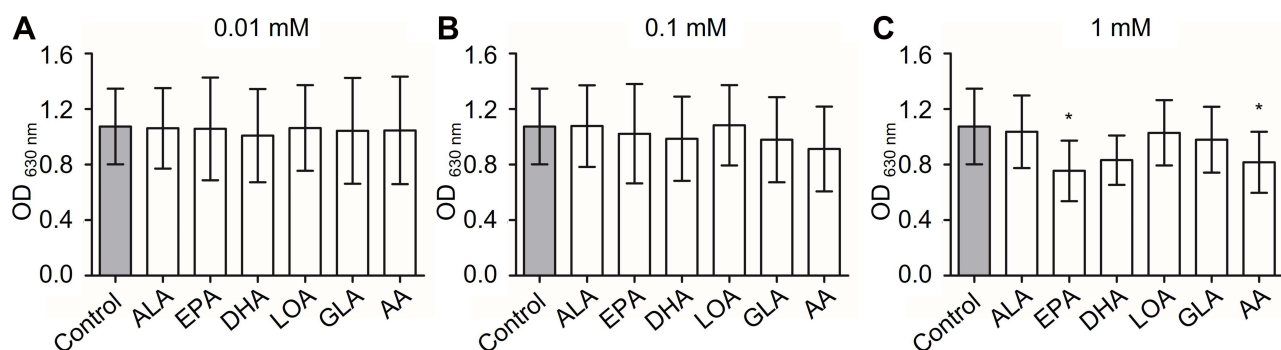


Figure 5 Eradication effect of essential fatty acids (EFAs) on mature biofilm of *C. albicans*. (A) 0.01 mM EFAs. (B) 0.1 mM EFAs. (C) 1 mM EFAs. The values shown are means \pm standard deviations. An asterisk denotes a statistically significant difference compared to the untreated control ($P < 0.05$).

Table 2 The MICs of Fluconazole in Combination with EFAs Against *C. albicans* Strains

Strains	MIC ($\mu\text{g/mL}$)						
	FLU	FLU+ALA	FLU+EPA	FLU+DHA	FLU+LOA	FLU+GLA	FLU+AA
Y02	0.25	0.5	1	1	1	0.25	1
Y20	2	8	8	8	16	8	8
Y26	1	1	1	1	1	1	1
Y37	0.25	0.5	0.5	0.5	0.5	0.5	1
Y38	0.5	2	2	2	2	1	2
Y65	8	8	8	8	8	8	8
Y70	1	2	4	4	2	2	4
X06	0.5	2	2	2	4	1	2
X09	0.25	1	1	1	1	0.5	1
X52	1	4	4	4	4	2	4
X54	2	8	8	8	8	4	8
X55	0.25	1	1	1	1	0.5	1
P-value	-	0.005	0.005	0.005	0.005	0.007	0.005

Abbreviations: MIC, minimum inhibitory concentration; FLU, fluconazole; ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LOA, linoleic acid; GLA, γ -linolenic acid; AA, arachidonic acid.

Table 3 The MBICs of Fluconazole in Combination with EFAs Against *C. albicans* Strains

Strains	MBIC ($\mu\text{g/mL}$)						
	FLU	FLU+ALA	FLU+EPA	FLU+DHA	FLU+LOA	FLU+GLA	FLU+AA
Y02	1	2	4	4	4	2	4
Y20	2	2	2	2	1	2	4
Y26	4	2	<0.125	<0.125	<0.125	4	<0.125
Y37	1	2	2	2	0.5	1	2
Y38	0.5	0.5	<0.125	<0.125	<0.125	0.5	<0.125
Y65	8	8	<0.125	<0.125	<0.125	8	<0.125
Y70	1	2	2	<0.125	0.125	1	2
X06	0.5	2	<0.125	<0.125	<0.125	1	0.125
X09	0.25	0.5	0.5	0.5	0.25	0.5	1
X52	1	2	<0.125	<0.125	<0.125	1	0.125
X54	2	4	<0.125	<0.125	0.125	2	0.125
X55	1	2	4	4	4	2	4
P-value	-	0.094	0.533	0.247	0.075	0.066	1.000

Abbreviations: MBIC, minimum biofilm inhibitory concentration; FLU, fluconazole; ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LOA, linoleic acid; GLA, γ -linolenic acid; AA, arachidonic acid.

Table 4 The MBECs of Fluconazole in Combination with EFAs Against *C. albicans* Strains

Strains	MBEC ($\mu\text{g/mL}$)						
	FLU	FLU+ALA	FLU+EPA	FLU+DHA	FLU+LOA	FLU+GLA	FLU+AA
Y02	128	64	16	8	8	8	8
Y20	128	128	128	128	128	128	128
Y26	256	256	256	256	256	256	256
Y37	256	64	128	256	128	64	64
Y38	> 256	> 256	> 256	> 256	> 256	> 256	> 256
Y65	> 256	> 256	> 256	> 256	> 256	> 256	> 256
Y70	> 256	> 256	> 256	256	> 256	> 256	> 256
X06	128	128	128	128	128	128	128
X09	64	8	32	64	8	8	8
X52	> 256	> 256	> 256	> 256	> 256	> 256	> 256
X54	256	256	256	256	256	256	256
X55	256	256	256	64	32	256	256
P-value	-	0.109	0.109	0.109	0.068	0.109	0.109

Abbreviations: MBEC, minimum biofilm eradication concentration; FLU, fluconazole; ALA, α -linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LOA, linoleic acid; GLA, γ -linolenic acid; AA, arachidonic acid.

127/135) under the conditions of our study. This result is similar to the previously reported data by Monfredini et al,³⁰ Gharaghani et al,³¹ and Hacıoglu et al.³² However, different from our results, lower biofilm formation rates (51.2% and 23.9%) of *C. albicans* were reported by Marak et al³³ and Tulasidas et al.³⁴ This may be due to differences in culture conditions during biofilm formation. Weerasekera et al³⁵ compared the effect of three culture media on the biofilm formation of *C. albicans*. They found biofilm biomass was higher in RPMI 1640 followed by sabouraud dextrose broth (SDB) and yeast nitrogen base (YNB). Therefore, RPMI 1640 medium was used in the biofilm assays, which could induce high biofilm biomass and is beneficial for evaluating anti-biofilm agents. With the increasing attention to biofilm-related infections, there is a need to develop a standardized culture condition for the *C. albicans* biofilm to facilitate cross-comparison between laboratories.

Fluconazole is currently one of the most widely used antifungal drugs to treat *C. albicans* infections. Although *C. albicans* is not intrinsically resistant to fluconazole in the planktonic state, the biofilms of *C. albicans* exhibit inherent antifungal drug resistance.³⁶ Our investigation also confirmed that *C. albicans* biofilms exhibit enhanced resistance against fluconazole. The MIC values of fluconazole for most tested strains were $\leq 0.125 \mu\text{g/mL}$. For the *C. albicans* strains with strong biofilm formation ability, fluconazole could inhibit biofilm formation of most strains, the MBIC values were 0.25–8 $\mu\text{g/mL}$; but when biofilms were established, resistance to fluconazole was greatly enhanced, and the MBEC values were $\geq 64 \mu\text{g/mL}$, increased from 64- to 1024- fold compared with MIC values. The mechanisms of fluconazole resistance in *Candida* biofilms include the increased metabolic activity occurring in the early development of biofilm, the presence of extracellular matrix, and alterations in gene expression (such as the upregulation of CDR and MDR genes encoding azole resistance transporters).³⁷ Therefore, it is critical to search for novel antifungal agents which can efficiently inhibit biofilm formation and eradicate mature biofilm of *C. albicans*.

EFAs are polyunsaturated fatty acids that play crucial roles in regulating body homeostasis, such as regulating the antioxidant signaling pathway, modulating inflammatory processes, and influencing hepatic lipid metabolism and physiological responses of other organs.^{23,38} Because of their nature, fatty acids are easily used as food additives.

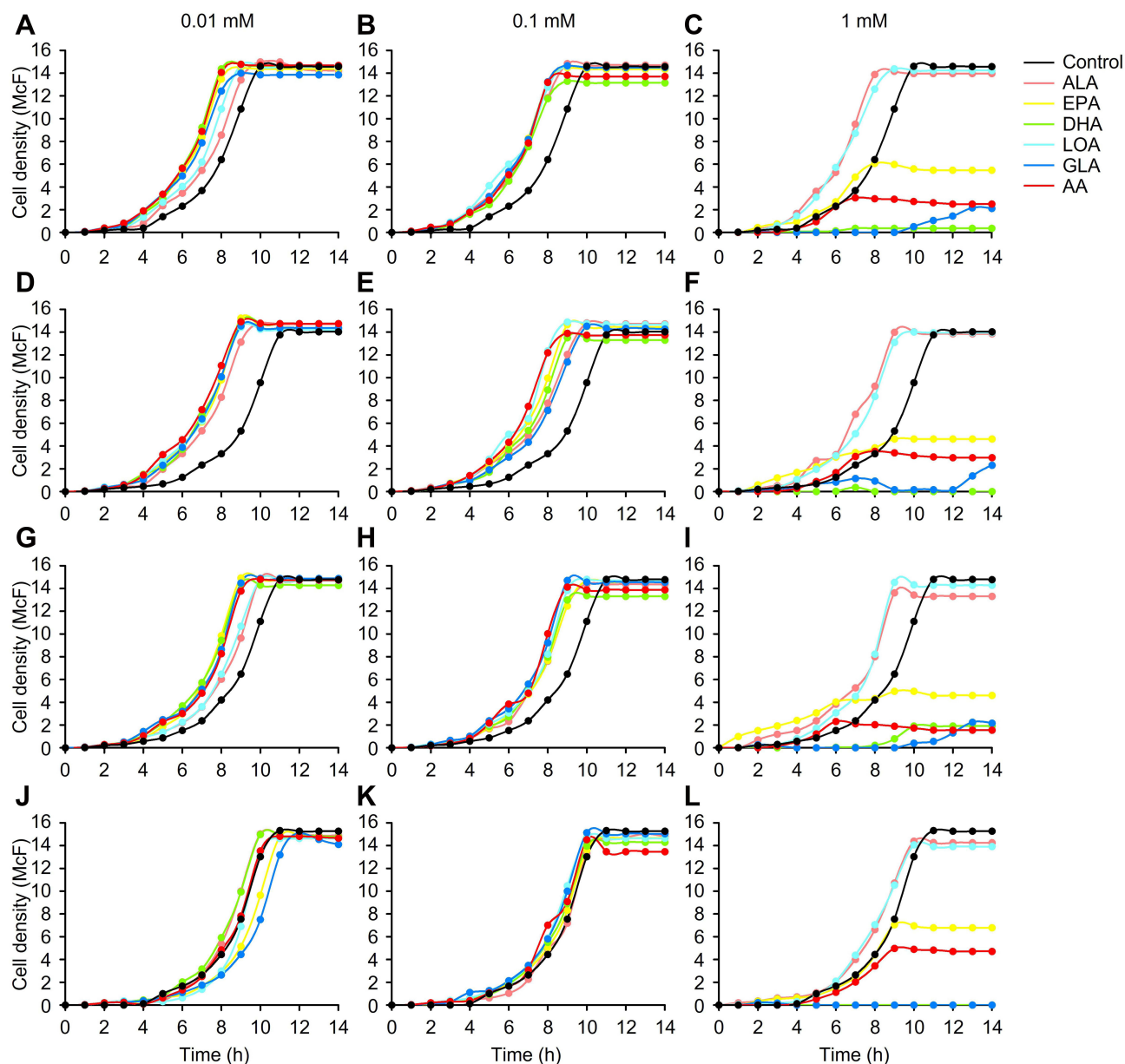


Figure 6 Growth curves of *C. albicans* strains in the absence (control) or the presence of different concentrations of essential fatty acids (EFAs). (A–C) *C. albicans* Y26, (D–F) *C. albicans* Y38, (G–I) *C. albicans* Y65, and (J–L) *C. albicans* X52. The McF values were determined every hour during 14 h.

EPAs can reduce the risk of various diseases, including cardiovascular disease,³⁹ cancer,⁴⁰ and Alzheimer's disease,⁴¹ among others.^{42,43} Several studies demonstrated that EFAs (eg, LOA, EPA, GLA, and AA) exerted antifungal properties against *Candida* spp.^{17–20} Nonetheless, few published studies have investigated the activities of EFAs on mature biofilms of *C. albicans*. Therefore, this study investigated the effect of EFAs, including ALA, EPA, DHA, LOA, GLA, and AA, against biofilm formation and mature biofilms of *C. albicans*.

Our results showed that other EFAs at 1 mM had inhibitory effects on biofilm formation except for LOA. The biofilm inhibition rates (%) ranged from 20.52% to 80.99% (LOA < ALA < AA < EPA < DHA < GLA) (Table S1). A recent study by Jamiu et al²⁰ also demonstrated LOA and GLA displayed significant inhibition of *C. krusei* biofilms. Both increased the susceptibility of *C. krusei* biofilm to fluconazole via induction of oxidative stress, cell membrane damage, and disruption of efflux pump activity. In this study, GLA exhibited a remarkable inhibitory effect against biofilm formation of *C. albicans*, indicating that GLA could be used as an alternative antifungal agent to prevent biofilm formation.

Furthermore, our research is the first to show that EPA and AA at 1 mM effectively eradicated mature biofilms of *C. albicans*, and the biofilm eradication rates were 29.95% and 24.14%. The biofilm eradication abilities of EPA and AA at 1 mM were similar to that of fluconazole at 64 µg/mL (26.15%). Although EFAs may not be as adequate as the high concentrations of fluconazole, pathogenic fungi are less likely to become resistant to antifungal EFAs.⁴⁴ Thus, EPA and AA have the potential to be used as antifungal agents for treating biofilm-associated infections.

In this study, our data also showed no synergistic effect between EFAs and fluconazole. EFAs at the low concentration (0.01 mM) even slightly increased MIC values of fluconazole. It might be due to low concentrations of EFAs promoting the growth of *C. albicans*. It should be noted that EPA, DHA, and GLA at 0.1 mM did not inhibit the growth rate of *C. albicans*, but did inhibit the biofilm formation of *C. albicans*. The underlying mechanisms for the antibiofilm potential of EFAs might involve affecting the adhering surface, changing cell-membrane fluidity, reducing extracellular polysaccharide or hyphae formation, and modulating quorum-sensing systems.⁴⁵

For the ω-3 fatty acids, the European Food Safety Authority suggests that long-term intake of EPA and DHA supplements up to about 5 g/day is safe;⁴⁶ and for ω-6 fatty acids, the United States Department of Agriculture indicates that the average adult intake of LOA is 17–20 g/day for men and 12–13 g/day for women.⁴⁷ Therefore, the maximum concentration of 1 mM (0.28–0.33 g/L) of EFAs used in the study is safe for patients with *C. albicans* infection.

Overall, this study indicates EPA and AA could serve as potential new antifungal agents for treating clinical infections caused by *C. albicans* biofilms.

Conclusion

In summary, the present study evaluated the antibiofilm activities of EFAs on *C. albicans* strains *in vitro*. GLA was found to inhibit biofilm formation by *C. albicans* markedly. Besides, EPA and AA exhibited better eradication effects on mature biofilms of *C. albicans*. These findings indicate GLA, EPA, and AA may be promising antifungal agents in managing *C. albicans*-related infections. Nevertheless, future studies are warranted to investigate the antibiofilm activity of EFAs on *C. albicans* *in vivo*, as well as the molecular mechanism of EFAs against the biofilm.

Ethical Approval

This study has been approved by the Ethics Committee of Beijing Chao-Yang Hospital, Capital Medical University (2021-2-26-3). Individual informed consent was waived by the ethics committee listed above because this study used the currently existing samples collected during routine medical care and did not pose any additional risks to the patients. This study was conducted in accordance with the Declaration of Helsinki.

Acknowledgments

The authors would like to thank all staff members of the Department of Infectious Diseases and Clinical Microbiology, Beijing Chao-Yang Hospital (Beijing, China), for their contribution to this work.

Supplementary Materials

[Table S1](#) The biofilm inhibition rates (%) of essential fatty acids (EFAs) and fluconazole against *C. albicans* strains;
[Table S2](#) The biofilm eradication rates (%) of essential fatty acids (EFAs) and fluconazole against *C. albicans* strains.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Talapko J, Juzbašić M, Matijević T, et al. *Candida albicans*-The virulence factors and clinical manifestations of infection. *J Fungi*. 2021;7(2):79. doi:10.3390/jof7020079
2. Turner SA, Butler G. The *Candida* pathogenic species complex. *Cold Spring Harb Perspect Med*. 2014;4(9):a019778. doi:10.1101/cshperspect.a019778
3. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. *Nat Rev Dis Primers*. 2018;4(1):18026. doi:10.1038/nrdp.2018.26

4. Pereira R, Dos Santos Fontenelle RO, de Brito EHS, de Moraes SM. Biofilm of *Candida albicans*: formation, regulation and resistance. *J Appl Microbiol*. 2021;131(1):11–22. doi:10.1111/jam.14949
5. Cavalheiro M, Teixeira MC. *Candida* Biofilms: threats, challenges, and promising strategies. *Front Med*. 2018;5:28. doi:10.3389/fmed.2018.00028
6. Wall G, Montelongo-Jauregui D, Vidal Bonifacio B, Lopez-Ribot JL, Uppuluri P. *Candida albicans* biofilm growth and dispersal: contributions to pathogenesis. *Curr Opin Microbiol*. 2019;52:1–6. doi:10.1016/j.mib.2019.04.001
7. Sustr V, Foessleitner P, Kiss H, Farr A. Vulvovaginal candidosis: current concepts, challenges and perspectives. *J Fungi*. 2020;6(4):267. doi:10.3390/jof6040267
8. Rodriguez-Cerdeira C, Gregorio MC, Molares-Vila A, et al. Biofilms and vulvovaginal candidiasis. *Colloids Surf B Biointerfaces*. 2019;174:110–125. doi:10.1016/j.colsurfb.2018.11.011
9. Rodriguez-Cerdeira C, Martínez-Herrera E, Camero-Gregorio M, et al. Pathogenesis and clinical relevance of *Candida* biofilms in vulvovaginal candidiasis. *Front Microbiol*. 2020;11:544480. doi:10.3389/fmicb.2020.544480
10. Tortorano AM, Prgitano A, Morroni G, Brescini L, Barchiesi F. Candidemia: evolution of drug resistance and novel therapeutic approaches. *Infect Drug Resist*. 2021;14:5543–5553. doi:10.2147/IDR.S274872
11. Xiao Z, Wang Q, Zhu F, An Y. Epidemiology, species distribution, antifungal susceptibility and mortality risk factors of candidemia among critically ill patients: a retrospective study from 2011 to 2017 in a teaching hospital in China. *Antimicrob Resist Infect Control*. 2019;8(1):89. doi:10.1186/s13756-019-0534-2
12. Vitális E, Nagy F, Tóth Z, et al. *Candida* biofilm production is associated with higher mortality in patients with candidaemia. *Mycoses*. 2020;63(4):352–360. doi:10.1111/myc.13049
13. Tascini C, Sozio E, Corte L, et al. The role of biofilm forming on mortality in patients with candidemia: a study derived from real world data. *Infect Dis*. 2018;50(3):214–219. doi:10.1080/23744235.2017.1384956
14. Soldini S, Posteraro B, Vella A, et al. Microbiologic and clinical characteristics of biofilm-forming *Candida parapsilosis* isolates associated with fungaemia and their impact on mortality. *Clin Microbiol Infect*. 2018;24(7):771–777. doi:10.1016/j.cmi.2017.11.005
15. Sasani E, Khodavaivis S, Rezaei S, Salehi M, Yadegari MH. The relationship between biofilm formation and mortality in patients with *Candida tropicalis* candidemia. *Microb Pathog*. 2021;155:104889. doi:10.1016/j.micpath.2021.104889
16. Casillas-Vargas G, Ocasio-Malavé C, Medina S, et al. Antibacterial fatty acids: an update of possible mechanisms of action and implications in the development of the next-generation of antibacterial agents. *Prog Lipid Res*. 2021;82:101093. doi:10.1016/j.plipres.2021.101093
17. Thibane VS, Ells R, Hugo A, et al. Polyunsaturated fatty acids cause apoptosis in *C. albicans* and *C. dubliniensis* biofilms. *Biochim Biophys Acta*. 2012;1820(10):1463–1468. doi:10.1016/j.bbagen.2012.05.004
18. Kuloyo O, Fourie R, Cason E, Albertyn J, Pohl CH. Transcriptome analyses of *Candida albicans* biofilms, exposed to arachidonic acid and fluconazole, indicates potential drug targets. *G3*. 2020;10(9):3099–3108. doi:10.1534/g3.120.401340
19. Kim YG, Lee JH, Park JG, Lee J. Inhibition of *Candida albicans* and *Staphylococcus aureus* biofilms by centipede oil and linoleic acid. *Biofouling*. 2020;36(2):126–137. doi:10.1080/08927014.2020.1730333
20. Jamiu AT, Albertyn J, Sebolai O, Gcilitshana O, Pohl CH. Inhibitory effect of polyunsaturated fatty acids alone or in combination with fluconazole on *Candida krusei* biofilms *in vitro* and in *Caenorhabditis elegans*. *Med Mycol*. 2021;59(12):1225–1237. doi:10.1093/mmy/myab055
21. Shareck J, Nantel A, Belhumeur P. Conjugated linoleic acid inhibits hyphal growth in *Candida albicans* by modulating Ras1p cellular levels and downregulating TEC1 expression. *Eukaryot Cell*. 2011;10(4):565–577. doi:10.1128/EC.00305-10
22. Thibane VS, Kock JL, Ells R, van Wyk PW, Pohl CH. Effect of marine polyunsaturated fatty acids on biofilm formation of *Candida albicans* and *Candida dubliniensis*. *Mar Drugs*. 2010;8(10):2597–2604. doi:10.3390/md8102597
23. Saini RK, Keum YS. Omega-3 and omega-6 polyunsaturated fatty acids: dietary sources, metabolism, and significance - A review. *Life Sci*. 2018;203:255–267. doi:10.1016/j.lfs.2018.04.049
24. Wayne P. *Clinical and Laboratory Standards Institute: Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. 4th ed. CLSI standard M27; 2017.
25. Gulati M, Lohse MB, Ennis CL, et al. *In vitro* culturing and screening of *Candida albicans* biofilms. *Curr Protoc Microbiol*. 2018;50(1):e60. doi:10.1002/cpmc.60
26. El-Houssaini HH, Elnabawy OM, Nasser HA, Elkhatib WF. Correlation between antifungal resistance and virulence factors in *Candida albicans* recovered from vaginal specimens. *Microb Pathog*. 2019;128:13–19. doi:10.1016/j.micpath.2018.12.028
27. Wang Y, Pei Z, Lou Z, Wang H. Evaluation of anti-biofilm capability of cordycepin against *Candida albicans*. *Infect Drug Resist*. 2021;14:435–448. doi:10.2147/IDR.S285690
28. Wei M, Wang P, Wang S, Yang C, Gu L. HB&L system for rapid phenotypic detection of clinical carbapenem-resistant *Enterobacteriales* isolates. *J Glob Antimicrob Resist*. 2021;26:272–278. doi:10.1016/j.jgar.2021.02.036
29. Koehler P, Stecher M, Cornely OA, et al. Morbidity and mortality of candidaemia in Europe: an epidemiologic meta-analysis. *Clin Microbiol Infect*. 2019;25(10):1200–1212. doi:10.1016/j.cmi.2019.04.024
30. Monfredini PM, Souza ACR, Cavalheiro RP, Siqueira RA, Colombo AL. Clinical impact of *Candida* spp. biofilm production in a cohort of patients with candidemia. *Med Mycol*. 2018;56(7):803–808. doi:10.1093/mmy/myx133
31. Gharaghani M, Rezaei-Matehkolaei A, Hardani AK, Zarei Mahmoudabadi A. Pediatric candiduria, epidemiology, genotype distribution and virulence factors of *Candida albicans*. *Microb Pathog*. 2021;160:105173. doi:10.1016/j.micpath.2021.105173
32. Hacıoğlu M, Guzel CB, Savage PB, Tan ASB. Antifungal susceptibilities, *in vitro* production of virulence factors and activities of ceragenins against *Candida* spp. isolated from vulvovaginal candidiasis. *Med Mycol*. 2019;57(3):291–299. doi:10.1093/mmy/myy023
33. Marak MB, Dhanashree B. Antifungal susceptibility and biofilm production of *Candida* spp. isolated from clinical samples. *Int J Microbiol*. 2018;2018:7495218. doi:10.1155/2018/7495218
34. Tulasidas S, Rao P, Bhat S, Manipura R. A study on biofilm production and antifungal drug resistance among *Candida* species from vulvovaginal and bloodstream infections. *Infect Drug Resist*. 2018;11:2443–2448. doi:10.2147/IDR.S179462
35. Weerasekera MM, Wijesinghe GK, Jayarathna TA, et al. Culture media profoundly affect *Candida albicans* and *Candida tropicalis* growth, adhesion and biofilm development. *Mem Inst Oswaldo Cruz*. 2016;111(11):697–702. doi:10.1590/0074-02760160294
36. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal drug resistance: molecular mechanisms in *Candida albicans* and beyond. *Chem Rev*. 2021;121(6):3390–3411. doi:10.1021/acs.chemrev.0c00199

37. de Barros PP, Rossoni RD, de Souza CM, Scorzoni L, Fenley JC, Junqueira JC. *Candida* biofilms: an update on developmental mechanisms and therapeutic challenges. *Mycopathologia*. 2020;185(3):415–424. doi:10.1007/s11046-020-00445-w
38. Djuricic I, Calder PC. Beneficial outcomes of omega-6 and omega-3 polyunsaturated fatty acids on human health: an update for 2021. *Nutrients*. 2021;13(7):2421. doi:10.3390/nu13072421
39. Abdelhamid AS, Brown TJ, Brainard JS, et al. Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease. *Cochrane Database Syst Rev*. 2018;7(7):CD003177. doi:10.1002/14651858.CD003177.pub3
40. Freitas RDS, Campos MM. Protective effects of omega-3 fatty acids in cancer-related complications. *Nutrients*. 2019;11(5):945. doi:10.3390/nu11050945
41. Dong X, Li S, Chen J, Li Y, Wu Y, Zhang D. Association of dietary ω -3 and ω -6 fatty acids intake with cognitive performance in older adults: national Health and nutrition examination Survey (NHANES) 2011–2014. *Nutr J*. 2020;19(1):25. doi:10.1186/s12937-020-00547-7
42. Christen WG, Schaumberg DA, Glynn RJ, Buring JE. Dietary ω -3 fatty acid and fish intake and incident age-related macular degeneration in women. *Arch Ophthalmol*. 2011;129(7):921–929. doi:10.1001/archophthalmol.2011.34
43. Shahidi F, Ambigaipalan P. Omega-3 polyunsaturated fatty acids and their health benefits. *Annu Rev Food Sci Technol*. 2018;9(1):345–381. doi:10.1146/annurev-food-111317-095850
44. Guimarães A, Venâncio A. The potential of fatty acids and their derivatives as antifungal agents: a review. *Toxins*. 2022;14(3):188. doi:10.3390/toxins14030188
45. Kumar P, Lee JH, Beyenal H, Lee J. Fatty acids as antibiofilm and antivirulence agents. *Trends Microbiol*. 2020;28(9):753–768. doi:10.1016/j.tim.2020.03.014
46. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific opinion on the tolerable upper intake level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA J*. 2012;10(7):2815.
47. U.S. Department of Agriculture. Agricultural Research Service. Nutrient intakes from food: mean amounts consumed per individual, by gender and age. Available from: <http://www.ars.usda.gov/ba/bhnrc/fsrg>. Accessed April 25, 2014.

Infection and Drug Resistance

Dovepress

Publish your work in this journal

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the development and spread of resistance. The journal is specifically concerned with the epidemiology of antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/infection-and-drug-resistance-journal>