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# Impact of Randomly Methylated Cyclodextrins on *Candida albicans*: Biofilm Formation, Morphogenesis and Oxidative Stress

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**Abstract**

The challenges related to biofilm-associated infections and diseases have prompted scientists to identify the factors responsible for the formation of biofilms and to develop strategies aimed at decreasing biofilm-formation capacity.

The present study aimed to evaluate the effect of randomly methylated cyclodextrin derivatives, at concentrations ranging from 32 nM to 12.5 mM, on the capacity of *Candida albicans* to form biofilms at 37 °C over a period of 24 h.

This study provides novel insights into how randomly methylated *α*-cyclodextrin (RAMEA), randomly methylated *β*-cyclodextrin (RAMEB), and randomly methylated *γ*-cyclodextrin (RAMEG) can modulate *C. albicans* biofilm formation. Using the crystal violet assay and the XTT reduction assay, we consistently demonstrated that RAMEA and RAMEG have a clear, concentration-dependent inhibitory effect on both the total biofilm biomass and the metabolic activity of the cells associated with these biofilms. RAMEB exhibited a biphasic effect: low to moderate concentrations significantly reduced biofilm formation, while higher doses unexpectedly resulted in increased biofilm formation. Microscopic analysis revealed that elevated cyclodextrin concentrations induced hyphal formation. Optical density measurements and a membrane permeability assay indicated that none of the cyclodextrins had a notable cytotoxic effect or damaged the cell membrane. Moreover, elevated intracellular ROS levels were detected, suggesting a potential stress-inducing effect. These findings enhance our understanding of the complex interactions between cyclodextrin derivatives and fungal cells, underscoring their potential as biofilm-modulating agents.

**Keywords**

antifungal activity, biofilm formation, *Candida albicans*, cyclodextrins

## 1 Introduction

*Candida* species are commonly found in the healthy human body, including the oral cavity, gastrointestinal tract, and urogenital organs. These opportunistic pathogenic microorganisms exhibit remarkable adaptability. Through their virulence factors, these otherwise harmless fungi can easily become pathogenic [1, 2]. In most clinical situations, among these species, *C. albicans* is the main cause of candidiasis [3, 4].

The ability of *Candida albicans* to undergo morphological transitions and form biofilms is crucial to its pathogenesis [4, 5]. Most infections caused by this pathogen are associated with the development of biofilms on host tissues or medical devices, contributing to elevated mortality rates. Although microorganisms have frequently been studied in free-floating (planktonic) cultures or as colonies grown on solid media, it is now widely accepted that biofilms represent the preferred and most physiologically relevant mode of growth for most microbial species [6–8].

Cells embedded in biofilms have demonstrated a high level of tolerance to antimicrobial therapy. As a result, *C. albicans* biofilms show significantly lower susceptibility to current antifungal agents compared to planktonic cells. The cells within the biofilm differ from free-floating cells in several ways. For example, certain enzymes secreted by the cells, such as proteases, are produced in higher quantities within biofilm-associated cells than those in planktonic cells, leading to different protein degradation patterns between the two cell types [9]. Yeast-like

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cells released from biofilms exhibit increased infectivity and enhanced adherence to various surfaces compared to conventional planktonic cells, facilitating the easier formation of new biofilms [10].

One of the primary mechanisms that regulates biofilm formation is quorum sensing (QS), a cell-to-cell communication system that regulates gene expression in response to cell density. QS plays a crucial role in the development and maintenance of *C. albicans* biofilms [11, 12].

Developing efficient strategies to combat biofilms that are highly resilient and resistant presents a considerable challenge, emphasizing the need for research focused on preventing the formation of such biofilms [5, 7, 13, 14].

Cyclodextrins (CDs) are cyclic oligosaccharides that have attracted great attention in recent decades due to their unique structure and polarity. These compounds have found broad application across diverse fields, including pharmaceutical sciences [15–17].

Their ability to form inclusion complexes with poorly soluble antifungal agents can enhance the drugs' aqueous solubility, stability, and bioavailability. Moreover, CDs may improve antifungal efficacy and offer potential advantages in the treatment of fungal infections [18–24].

The use of native CDs is often limited due to certain inherent properties; thus, chemically modified derivatives are frequently utilized instead. Derivatization can enhance important characteristics such as solubility, molecular selectivity, and the stability of inclusion complexes, while also potentially reducing toxicity [25–27]. Among the native CDs, β-cyclodextrin (composed of seven glucose units) is particularly poorly soluble in water. Methylation modifies the hydrophilic–hydrophobic balance of the molecule, resulting in significantly improved water solubility — up to several hundred times greater in the case of methylated derivatives such as randomly methylated β-cyclodextrin (RAMEB) [28, 29]. CDs have proven effective as auxiliary agents in combination with various active compounds to combat bacterial and fungal biofilms [22, 30]. Additionally, some studies suggest that CDs can directly modulate biofilm formation, even without the presence of co-administered bioactive substances [31–33].

In our previous research [33], we investigated the concentration-dependent effects of native CDs and various CD derivatives within a range of 0.5–12.5 mM, aiming to identify mechanisms to reduce biofilm formation. Our findings revealed that CDs could exert both stimulatory and inhibitory effects on biofilm formation, depending on their structural characteristics and applied concentrations. Notably, the randomly methylated derivatives exhibited significant antifungal activity, with a marked reduction in the biofilm-forming ability of *Candida albicans*. Even the lowest tested concentration (0.5 mM) of all three randomly methylated CDs examined effectively inhibited the biofilm-forming ability of *Candida albicans*. This study extends the investigation to a broader concentration range to more thoroughly characterize the impact of CDs on biofilm development and explore the underlying mechanisms of inhibition. Furthermore, we investigated the effects of randomly methylated CDs on the morphological transitions of *Candida albicans*, monitoring fungal growth throughout. To explore potential causes, membrane integrity studies were performed, and the production of reactive oxygen species (ROS) was monitored.

## 2 Methods

### 2.1 Strain and tested cyclodextrin molecules

The tests were conducted using the *C. albicans* strain DSMZ 1386 (ATCC 10231) which has been shown to effectively form biofilms in previous studies. The strain was maintained on solid YPD agar slants composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. For the testing, a single loopful of the maintained culture was inoculated into 30 mL of sterile liquid YPD medium and incubated at 30 °C for 16 h under continuous agitation at 150 rpm. Under these conditions, the yeast primarily grows in budding-yeast form.

Following incubation, cells were harvested by centrifugation at 4000 × g, washed twice with sterile phosphate-buffered saline (PBS), and resuspended in RPMI-1640 medium (Capricorn Scientific) to promote biofilm formation. The cell density was standardized to an optical density ($OD_{600}$) of 0.2 to ensure consistent cell input across assays.

The tested CD molecules (Table 1) were obtained from CycloLab Ltd. For the experiments, the CD molecules

**Table 1** Abbreviations (with the degree of substitution), average molecular formula and molecular mass of tested CDs

| Name | Abbreviation | Average molecular formula | Molecular mass |
|---|---|---|---|
| Randomly methylated *α*-cyclodextrin | RAMEA (11) | $C_{36}H_{60-n}O_{30}\cdot(CH_3)_n$ | 1127 |
| Randomly methylated *β*-cyclodextrin | RAMEB (12) | $C_{42}H_{70-n}O_{35}\cdot(CH_3)_n$ | 1303 |
| Randomly methylated *γ*-cyclodextrin | RAMEG (12) | $C_{48}H_{80-n}O_{40}\cdot(CH_3)_n$ | 1465 |

were suspended in distilled water and sterilized through autoclaving. A dilution series was then prepared, covering a concentration range of 0.0008–50 mM.

### 2.2 Biofilm measurement

Biofilm formation by *Candida albicans* was assessed using two complementary quantitative methods: the crystal violet (CV) staining assay and the XTT tetrazolium salt reduction assay. These methods measure total biomass and metabolic activity, respectively.

#### 2.2.1 Crystal violet assay

The CV assay followed a modified version of the O'Toole method [34], as previously described [32]. In brief, 50 µL of the sample and 150 µL of the cell suspension were pipetted into sterile, flat-bottom 96-well polystyrene microplates and incubated at 37 °C for 24 h. After incubation, the supernatant was carefully removed, and the wells were rinsed twice with water to eliminate loosely attached cells. To fix the adherent biofilm, 250 µL of methanol was added to each well and incubated for 15 min. Following this, the methanol was removed, and the plates were air-dried under laminar flow. Staining was performed by adding 250 µL of 0.1% (w/v) aqueous CV solution to each well, followed by a 15-min incubation. After discarding the dye, the wells were gently washed to remove any excess stain while minimizing disruption of the biofilm. The fixed dye was then solubilized using 250 µL of 30% (v/v) acetic acid. Finally, a 250 µL aliquot of the resulting solution was transferred to a new microplate, and absorbance was measured at 544 nm using a Fluostar Optima plate reader (BMG Labtech, Germany).

#### 2.2.2 XTT reduction assay

The XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide] assay was performed in parallel under the same culture conditions as for the CV assay to measure the metabolic activity of cells in the biofilm. Following biofilm development, wells were gently washed two times with water to remove nonadherent cells. A freshly prepared XTT-menadione solution was used to assess metabolic activity. XTT (Sigma-Aldrich Inc. Budapest, Hungary) was dissolved in sterile PBS at a concentration of 1 mg/mL, while menadione was prepared at a concetration of 0.4 mM in acetone. Both solutions were filter sterilized (0.22 µm) and mixed at a 12:1 (v/v) ratio immediately before use. For the assay, 30 µL of the XTT-menadione mixture and 250 µL of PBS were added to each well. Plates were incubated in the dark at 37 °C for 1 h. Following incubation, 100 µL of the colored formazan produced by the reduction of XTT was transferred to a new plate, and the absorbance was read at 450 nm using a DIALAB ELx800 microplate reader (Dialab GmbH, Wiener Neudorf, Austria).

### 2.3 Growth assessment by optical density determination

To determine potential cytotoxic effects exerted by the applied CDs, optical density (OD) measurements were performed at 630 nm. The OD was measured using a DIALAB ELx800 ELISA microplate reader, both immediately after setting up the plates and following the incubation periods.

### 2.4 Microscopic evaluation of morphological changes

After 24 h of incubation and CV staining, microscopic analysis was performed to investigate potential morphological alterations in *Candida albicans* cells exposed to CDs. Imaging was performed using a Nikon Eclipse SI microscope, equipped with a TrueChrome 4K Pro digital camera and Mosaic™ V2.4 software (Auro-Science Consulting Kft., Budapest, Hungary) [35]. Images were captured at 200x magnification for a microscopic evaluation of the morphological changes induced by CDs.

### 2.5 Measurement of ROS production

ROS were detected using 2',7'-dichlorofluorescin diacetate (DCFDA) (Sigma-Aldrich Inc., Budapest, Hungary), following a measurement of fluorescence based on a previously described protocol [36] with modifications. Briefly, the cells were exposed to different concentrations of CDs at 37 °C for 24 h in YPD. The cells were then harvested by centrifugation, washed twice, and resuspended in PBS. Subsequently, 150 µL of the cell suspension was transferred to a black flat-bottom 96-well microplate, and 20 µL of 20 µM DCFDA was added to the wells, followed by 30-min incubation. The fluorescence intensity (with excitation and emission of 485 and 520 nm), which directly indicates ROS levels, was measured with a Fluostar Optima (BMG Labtech, Germany) microplate reader.

### 2.6 Membrane permeability assay with EtBr

The membrane permeability assay was performed according to a previously published protocol with modifications [37]. An overnight culture of *C. albicans* was washed twice with PBS and diluted to OD600 = 0.8 in PBS, which contained 10 µM ethidium bromide (EtBr, Sigma-Aldrich Inc. Budapest, Hungary) and 5 mM glucose. Subsequently,

1.5 ml of the fungal suspension was incubated with 0.5 mL of the tested CDs or control for 60 min at 30 °C, 400 RPM. To prepare the heat-killed cells, the sample was incubated at 80 °C for 10 min. Then, 100 µL of the samples were transferred into a 96-well black microplate and fluorescence was measured with Fluostar Optima fluorometer with the excitation and emission wavelengths set to 544 and 590 nm, respectively. The slit width was 10 nm.

### 2.7 Statistical analysis

All experimental data were statistically evaluated by analysis of variance (ANOVA) using Statistica 13.1 software (TIBCO Software, Inc., Palo Alto, USA) [38] to assess significant differences between treatments. Pairwise comparisons between different CD concentrations were conducted using Fisher's least significant difference (LSD) test, or alternatively, the Newman–Keuls procedure was applied when the criteria of variance homogeneity were not fulfilled. Columns annotated with the same letter do not differ significantly at the $p < 0.05$ significance level. All statistical tests were performed with a confidence level set at $p < 0.05$.

A dose–response analysis was conducted by Origin® 2018 (OriginLab, Northampton, MA, USA), employing a sigmoidal fit dose-response function to determine effective concentrations for biofilm inhibition. To determine these effective concentration values (EC), sigmoidal curves were fitted to the inhibition percentage values calculated from the measurement data compared to the control.

The concentrations of the examined CDs that result in 20% and 50% inhibition of the observed endpoint are denoted by EC20 and EC50, respectively.

## 3 Results

The aim of this study was to evaluate the effect of randomly methylated CD derivatives on biofilm formation. To achieve this, two complementary methods were used: the CV staining technique, which quantifies total biofilm biomass, and the XTT reduction assay, which assesses the metabolic activity of cells embedded in the biofilm. The graphs presented illustrate relative biofilm formation, calculated as the ratio of the OD measured at 544 nm after CV staining to the OD measured at 630 nm before staining in the treated wells was divided by the same ratio obtained for the untreated control wells.

Fig. 1 illustrates the impact of randomly methylated $\alpha$-CD (RAMEA) within the concentration range of 32 nM to 12.5 mM.

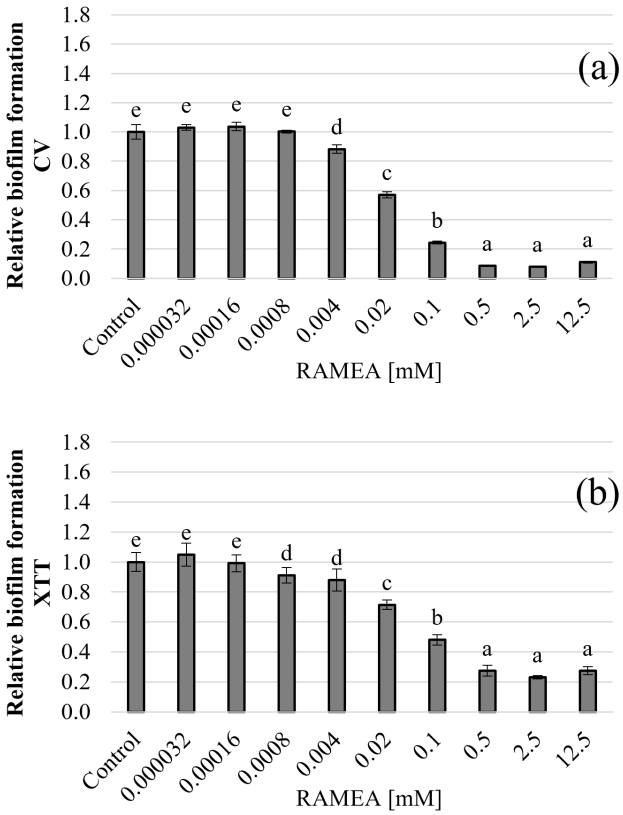


**Fig. 1** Effect of increasing concentration of RAMEA on biofilm formation after 24 h of incubation: results obtained using (a) the CV staining method are presented on the top, whereas (b) data derived from the XTT reduction assay are shown on the bottom. Values sharing the same letter do not differ significantly from each other at $p < 0.05$.

Both assays revealed that RAMEA exerted a concentration-dependent inhibitory effect on biofilm formation, when compared with the untreated control. As indicated by the identical letters above the bars, the lower concentrations up to 16 nM did not cause significant changes in either biomass or metabolic activity ($p > 0.05$). However, at higher concentrations, a significant decrease in both total biomass and metabolic activity is observed. At the highest tested concentrations, the inhibition exceeded 90% in the CV assay and reached approximately 70% in the XTT assay, clearly demonstrating the strong biofilm-suppressive potential of RAMEA at elevated doses. In the case of RAMEB (Fig. 2), a similar concentration-dependent biofilm inhibition was observed up to a concentration of 0.5 mM. Below this threshold, both the total and the metabolic activity decreased in a concentration-dependent manner compared to the untreated control. However, at concentrations higher than 0.5 mM, this trend was reversed. Notably, the biofilm biomass began to increase again, and at the highest tested concentration of 12.5 mM,

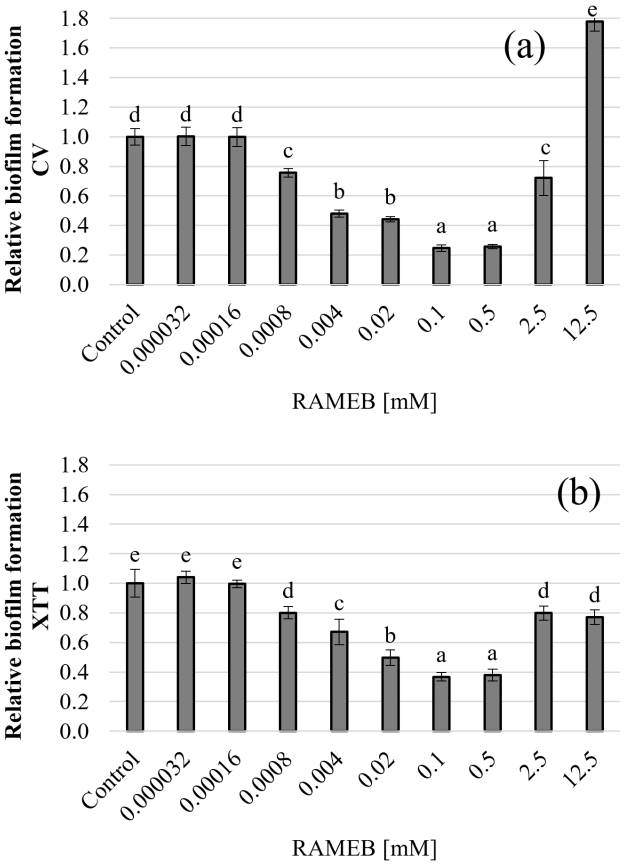


**Fig. 2** Effect of increasing concentration of RAMEB on biofilm formation after 24 h of incubation: results obtained using (a) the CV staining method are presented on the top, whereas (b) data derived from the XTT reduction assay are shown on the bottom. Values sharing the same letter do not differ significantly from each other at $p < 0.05$.

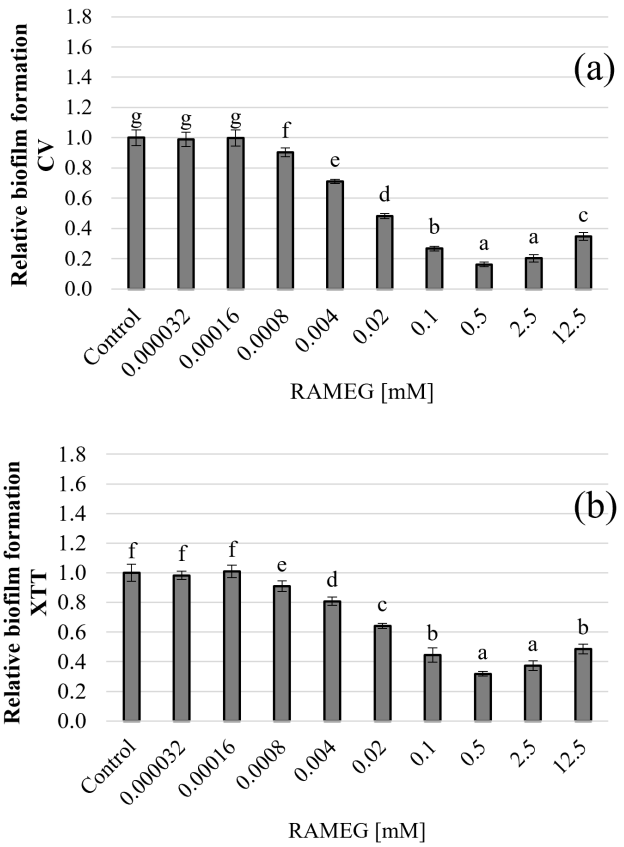


**Fig. 3** Effect of increasing concentration of RAMEG on biofilm formation after 24 h of incubation: results obtained using (a) the CV staining method are presented on the top, whereas (b) data derived from the XTT reduction assay are shown on the bottom. Values sharing the same letter do not differ significantly from each other at $p < 0.05$.

the CV assay revealed an approximately 80% increase in biofilm biomass relative to the control.

The unexpected biofilm-promoting effect observed at high RAMEB concentrations suggests that there may be a threshold concentration above which the compound induces cellular responses that favor biofilm formation instead of suppression. In the case of randomly methylated γ-cyclodextrin (RAMEG) (Fig. 3), the most pronounced inhibitory effect on biofilm formation was also noted at a concentration of 0.5 mM. This resulted in a reduction of approximately 84% in the CV assay and 68% in the XTT assay compared to the control.

At concentrations higher than 0.5 mM, a slight increase in biofilm formation was detected However, even at the highest concentration tested (12.5 mM), the level of biofilm remained significantly lower than those of the untreated control. This indicates that RAMEG consistently exerts a strong anti-biofilm effect across all tested concentrations.

To evaluate the potential cytotoxic effects of the applied CDs, we measured changes in OD. The results, summarized in Table S1, show that the OD fluctuated by no more than 11% across all treatments. This suggests that the observed inhibition of biofilm formation was not accompanied by a significant reduction in microbial growth.

Based on the statistically determined $EC_{20}$ and $EC_{50}$ values in Table 2, the efficiency order of CDs regarding their inhibitory capacity on biofilms is as follows:

$$\text{RAMEA} < \text{RAMEG} < \text{RAMEB} \quad (1)$$

**Table 2** Effective concentrations causing 20% and 50% inhibition of biofilm formation capacity ($EC_{20}$, $EC_{50}$) and the lowest observed inhibitory concentration (LOIC) for RAMEA, RAMEB, RAMEG

| | $EC_{20}$, $EC_{50}$ and LOIC values [μM] | | | | | |
|---|---|---|---|---|---|---|
| | Biofilm formation capacity – CV staining | | | Biofilm viability – XTT reduction | | |
| | RAMEA | RAMEB | RAMEG | RAMEA | RAMEB | RAMEG |
| $EC_{20}$ | 8.6 | 0.8 | 4.0 | 17.3 | 1.8 | 7.0 |
| $EC_{50}$ | 28.3 | 3.2 | 15.3 | 73.9 | 8.4 | 36.2 |
| LOIC | 4.0 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |

LOIC refers to the lowest observed inhibitory concentration of CDs, which results in significant inhibition of biofilm formation as determined by ANOVA.

To evaluate the impact of randomly methylated CDs on fungal morphology, we captured microscopic images presented in Fig. 4. The images clearly illustrate that in samples where biofilm formation was reduced due to CD treatment, there was also a marked decrease in the number of visible cells. Higher concentrations of CD resulted in more hyphal structures, indicating that elevated CD levels may promote hyphal development.

In this study, we studied how the tested CDs affect the generation of ROS in *Candida albicans* cells. ROS are highly reactive and short-lived molecules, which makes their direct detection challenging. To estimate the level of ROS produced in this experiment, we used DCFDA, a fluorescent probe. An increase in fluorescence indicates overall oxidative activity, although it does not specify which reactive species are present. Based on this principle, we interpreted the fluorescence intensity (relative to control), shown in Fig. 5, to assess how the randomly methylated CDs influence intracellular ROS levels under the tested conditions.

The results indicate that all three tested CD derivatives led to a significant increase in intracellular ROS levels. Notably, RAMEG induced the highest ROS production; at the maximum tested concentration of 12.5 mM, the ROS level rose by approximately 140% compared to the untreated control. Additionally, this increase

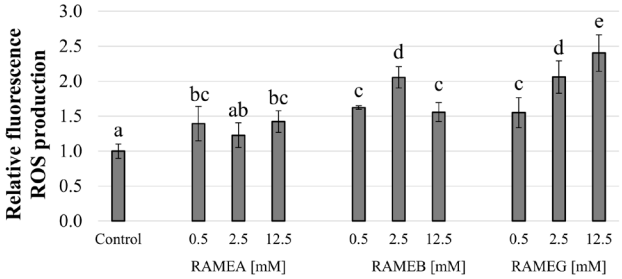


**Fig. 5** ROS measured in Candida albicans cells using the DCFDA assay. Values sharing the same letter do not differ significantly from each other at $p < 0.05$.

demonstrated a clear concentration-dependent trend, suggesting that higher concentrations of RAMEG are associated with greater oxidative stress within the fungal cells.

The effect on membrane integrity was also assessed using a membrane permeability assay (see Fig. S1). The results revealed that cells treated with randomly methylated CDs did not exhibit increased fluorescence signals compared to the untreated control, suggesting that these treatments did not result in a higher proportion of cells with damaged membranes.

## 4 Discussion

This study provides new insights into the potential of randomly methylated CD derivatives (RAMEA, RAMEB, and RAMEG) to modulate the formation of biofilms in *Candida albicans*. Two commonly used methods for evaluating biofilm formation in *Candida* species are the XTT assay, which

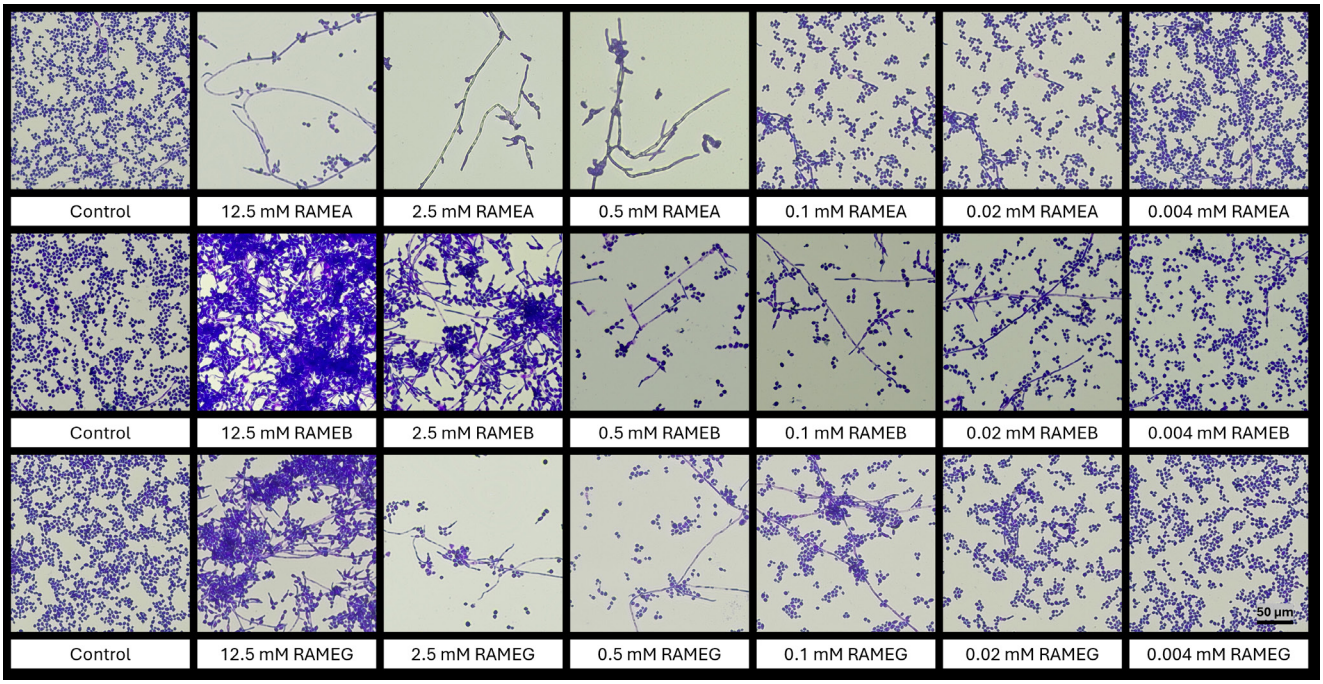


**Fig. 4** Biofilm formation of *Candida albicans* after staining with CV, observed with a Nikon Eclipse SI microscope at 200x magnification

measures cell viability, and the CV staining technique, which quantifies total biofilm biomass [34, 39]. Both methods were employed in this study. The results consistently demonstrate that RAMEA and RAMEG exert a concentration-dependent inhibitory effect on both total biofilm biomass and metabolic activity, with the strongest inhibition observed at higher doses. In contrast, RAMEB displayed different behavior: while low to moderate concentrations effectively reduced biofilm formation, higher doses unexpectedly stimulated biomass accumulation, suggesting a concentration threshold beyond which the compound may promote biofilm growth. The outcomes of the XTT and CV assays were consistent with each other with no major discrepancies observed, indicating that the cells within the biofilm remained viable. This aligns with previous studies [40], which reported a significant correlation between the results of these two methods, but we can also find studies where poor agreement was shown in *Candida* species [39].

Microscopic images confirmed these findings, showing a reduced number of cells where biofilm was inhibited. A notable finding was that CDs induced more pronounced hyphae formation than the control group at nearly all tested concentrations. The underlying mechanism for this result remains to be elucidated, but it may be related to the influence of CDs on the availability of signal molecules that influence biofilm formation. Additionally, the outcome may also be affected by potential interactions between CDs and medium components, leading to changes in nutrient availability.

Overall, these results indicate that randomly methylated CDs can interfere with the development of *C. albicans* biofilms and morphological transitions, although their effects are strongly dependent on chemical structure and concentration. Understanding these interactions could help in developing new strategies to control biofilm-related infections and improve antifungal treatments in the future.

Polymorphism, or the ability to undergo morphological transitions, is crucial for their adaptation to different environmental conditions. The ability to switch between yeast, hyphal, and pseudohyphal forms is fundamental to the virulence of the species, contributing to both survival and pathogenicity within the host. This transition is influenced by several factors, including temperature, pH, carbon dioxide concentration, and the presence of mammalian serum [41–43]. Several studies have shown that strains unable to form hyphae cause significantly milder symptoms, further supporting the role of polymorphism and biofilm formation in the pathogenesis of chronic or recurrent infections [44, 45].

It was also demonstrated that the randomly methylated CDs did not cause notable cytotoxic effects, as evidenced by OD measurements. Studies examining the effect of cavity size and substitution patterns on the cytotoxicity of CDs revealed that methyl-substituted CDs displayed similar cytotoxicity to their native counterparts [39].

To further investigate the effects of randomly methylated CDs on *Candida albicans*, we examined additional endpoints to elucidate potential underlying mechanisms. In our membrane integrity assays using EtBr, we observed no increase in fluorescence signal in CD-treated cells compared to the control group, indicating that membrane permeability was not notably altered by the CDs. However, the data suggested that all three types of CDs slightly slowed cell growth, as evidenced by the lower fluorescence signals compared to untreated controls. This indicates that there was a lower concentration of dead cells in these systems. This finding is further supported by the heat-killed control samples included in the assay, which show that the fluorescence intensity correlates directly with total cell count. Similarly, lower signals were detected in samples treated with CDs.

Additionally, we assessed the impact of randomly methylated CDs on intracellular ROS levels. Our results revealed a significant increase in ROS levels after 24 h treatment. This increase indicates that randomly methylated CDs induce cellular stress in *Candida albicans*. While our membrane integrity assays did not reveal significant changes in membrane permeability, it is plausible that these CDs alter the composition and fluidity of the plasma membrane or disturb the cell wall structure. Such modifications could interfere with nutrient and ion transport, potentially disrupting metabolic homeostasis and leading to oxidative stress within the cells. *C. albicans* is known to respond to various environmental challenges, including cell wall damage and exposure to antifungal agents, by increasing ROS production [46]. Therefore, the elevated ROS levels detected in our experiments suggest that, in addition to directly inhibiting biofilm formation, randomly methylated CDs may compromise cellular homeostasis through the induction of oxidative stress mechanisms.

## 5 Conclusion

Although the underlying mechanisms are still partly unexplored, our results clearly illustrate that randomly methylated CD derivatives are capable of influencing *Candida albicans* biofilm formation and can affect its morphological transitions. These cyclic oligosaccharides possess exceptional properties, as demonstrated by

their ability to reduce the infectivity of *Candida albicans* through the induction of cytotoxic effects and their impact on biofilm formation. This clearly opens up new directions for the research and development of antifungal agents.

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