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ORIGINAL RESEARCH

Evaluation of Anti-Biofilm Capability of Cordycepin Against *Candida albicans*

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Introduction: The opportunistic pathogen *Candida albicans* can form biofilms, resulting in drug resistance with great risk to medical treatment.

Methodology: We investigated the ability of *C. albicans* to form biofilms on different materials, as well as the inhibitory and eradicating effects of cordycepin on biofilm. The action mechanism of cordycepin against biofilm was studied by crystal violet staining, XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction method, phenol-sulfuric acid method, cellular superficial hydrophobicity (CSH) assay, and confocal laser scanning microscope observation. We also evaluated the acute toxicity of cordycepin in vivo.

Results: The results showed facile formation of biofilms by *C. albicans* on polypropylene. The 50% minimum inhibitory concentration (MIC_{50}) of cordycepin was 0.062 mg/mL. A concentration of 0.125 mg/mL significantly decreased biofilm formation, metabolic activity, secretion of extracellular polysaccharides, and relative CSH. Cordycepin could inhibit biofilm formation at low concentration without affecting fungal growth. In addition, cordycepin effectively eradicated 59.14% of mature biofilms of *C. albicans* at a concentration of 0.5 mg/mL. For acute toxicity, the LD_{50} (50% of lethal dose) of cordycepin was determined as higher than 500 mg/kg for mice.

Conclusion: The results of this study show that cordycepin significantly inhibited and eradicated biofilms by decreasing metabolic activity, the ratio of living cells, the hydrophobicity, and damaging the extracellular polysaccharides of biofilm. These findings should facilitate more effective application of cordycepin and suggest a new direction for the treatment of fungal infections.

Keywords: cordycepin, *Candida albicans*, biofilm, inhibition, eradication

Introduction

Candida albicans is normally commensal on human mucosal surfaces, but can become an opportunistic pathogen causing infections at various levels, ranging from superficial to life-threatening systemic infections.[1] At present, fluconazole, nystatin, amphotericin B, and echinocandin are mainly used for clinical treatment.[2–5] However, with few traditional antifungal drugs and the challenges of long-term use of these drugs, drug resistance is gradually increasing. The ability of *C. albicans* to form biofilms is an important factor in its resistance to treatment, and the resistance of fungal biofilms can be 1000 times higher than that of free microorganisms.[6] A biofilm is a microbial community that is embedded in a self-produced extracellular matrix and adsorbed on the surface of an object.[7] The extracellular matrix helps preserve the biofilm structure and protects cells from

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host defenses and drug attacks.[8,9] Given the effect of biofilm formation to promote drug resistance and the limitations of existing drugs, such as the substantial toxicity of amphotericin B, there is an urgent need for natural alternatives.[4]

Cordycepin, also known as 3′-deoxyadenosine, is a nucleoside analogue that is extracted from *Cordyceps militaris*.[10] Cordycepin is structurally similar to adenosine, but lacks the 3′-hydroxyl group, which increases its efficacy. Cordycepin has some biological activities such as antimicrobial, anti-tumor, anti-inflammatory, and immunomodulation.[11–14] The antimicrobial activity of cordycepin is broad-spectrum and can inhibit eight kinds of lactic acid bacteria and 11 kinds of intestinal bacteria.[15] Sugar and McCaffrey characterized the antifungal activity of cordycepin using in vivo experiments.[16] The antimicrobial mode of cordycepin has been studied, with the drug affecting multiple processes such as the uptake of cordycepin through the nucleoside transporter of *C. albicans*, followed by binding to hydrophobic bases or phosphate groups of DNA to alter the structure and function of the microbial nucleic acids, eventually leading to cell death.[17,18] However, most work has focused on the effects of cordycepin on planktonic microorganisms, with no investigation of the effect of cordycepin on *C. albicans* biofilm.[15,17] In this study, we investigated the effects of cordycepin on the formation and elimination of *C. albicans* biofilm and determined the action mechanism to enable more effective application of cordycepin.

Materials and Methods

Materials, Strains and Growth Conditions

Cordycepin (≥98%) and fluconazole (96%) were purchased from the Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The strains, *C. albicans, Candida tropicalis, Candida glabrata* and *Candida parapsilosis* were clinically isolated strains provided by the clinical laboratory of Wuxi No. 2 People's Hospital. Antifungal susceptibility test was also performed using *C. albicans* standard strains (BNCC 186382, ATCC 10231, and CMCC 98001). *C. albicans* BNCC 186382 was purchased from Beina Chuanglian Biotechnology Co., Ltd. (Beijing, China), and other standard strains were purchased from Guangdong Huankai Microbial Technology Co., Ltd. (Guangdong, China). The strains were identified by automatic microbial mass spectrometry detection system (bioMerieux, VITEX MS, France) and stored in a glycerol tube at −80°C. The strains were cultivated to the exponential phase in potato dextrose broth (PDB, Hangzhou Baisi Biotechnology Co., Ltd, Hangzhou, China) before use.

Determination of Minimum Inhibitory Concentration (MIC) of Cordycepin

The MIC was determined using the standard broth microdilution method as previously described in document M27 published by the Clinical and Laboratory Standards Institute.[19] One hundred microliters of drug solution was added to the first column of wells and then was twofold serial diluted in RPMI-1640 medium (Hangzhou Baisi Biotechnology Co., Ltd, Hangzhou, China), buffered with 165 mM 3-(N-morpholino) propanesulfonic acid (MOPS, Beijing Solarbio Science & Technology Co., Ltd, Beijing, China), pH 6.5, with initial concentrations of cordycepin and fluconazole of 2 and 0.064 mg/mL, respectively. Fungal suspensions were then diluted in RPMI-1640 medium and added to each well at a final concentration of 10^3 cells/mL. The well plate was incubated at 35°C for 48 h. The document M27 states that MIC endpoint of fluconazole and flucytosine is the lowest concentration that inhibited 50% of the fungal yeast growth in relation to control (MIC_{50}).[19] Flucytosine is a nucleotide-analogue antibiotic with a mechanism of disturbing protein and DNA synthesis.[20] Here, we considered MIC_{50} as the MIC endpoint of cordycepin due to the similar mechanism and structural characteristics of cordycepin and flucytosine.

Effects of Cordycepin on the Growth of *C. albicans*

The *C. albicans* suspensions were inoculated into 50 mL of RPMI-1640 medium to obtain a final concentration of 10^5–10^6 cells/mL, and different concentrations of cordycepin (0.16, 0.31, 0.062, and 0.125 mg/mL) were added at the same time. The mixture was incubated at 35°C and 150 rpm for 48 h. During this period, absorbance changes at 600 nm were recorded at regular intervals.[21]

The Ability of *C. albicans* to Form Biofilms on Different Materials

Crystal violet staining was used in this assay to evaluate the effect of different materials on the biofilm formation of *C. albicans*.[22] Stainless steel, plastic polypropylene and glass were divided into 25 x 25 x 1 mm square pieces

and soaked in 75% ethanol overnight. The materials and the cell suspensions with a final concentration of 10^6 cells/mL were incubated in a 6-well plate at 35°C for 24 h without shaking to form a biofilm.[23] After that, the well plate was washed three times by phosphate-buffered saline (PBS, pH=6.5). The attached cells were fixed by drying at 60°C for 30 min, and then, 4 mL of 0.1% (w/v) crystal violet was added and left for 5 min. Excess staining solution was removed by washing three times with PBS. The stain retained by the biofilm was dissolved in 4 mL of 33% (v/v) glacial acetic acid. After 10 min, the solution in the treated plate was transferred to a clean 96-well plate, and the OD value was measured at 595 nm.

Analysis of the Effects of Cordycepin on the Formation of *C. albicans* Biofilm

The cell suspensions were co-cultured with different concentrations of cordycepin, and the final concentrations of cordycepin were 0.16, 0.031, 0.062, and 0.125 mg/mL, with no addition of cordycepin as control. Then, the well plates were incubated at 35°C for 24 h. The final data were calculated according to the formula:

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

Analysis of the Effects of Cordycepin on the Pre-Formed Biofilms

The mature biofilms of *C. albicans* were pre-formed by adding only cell suspensions and culturing at 35°C for 24h. After incubation, the non-adherent cells were removed by washing with PBS, and RPMI-1640 medium containing different concentrations of cordycepin was added to the well plate, in which the final concentration of cordycepin was 0.62, 0.125, 0.25, and 0.5 mg/mL, respectively. The well plate was covered with its lid and incubated at 35°C for an additional 4 h. The final data were calculated according to the formula:

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

Quantification of Biofilm

Quantification of biofilm was also analyzed by crystal violet staining assay.[22] After incubated for an appropriate time, the 96-well plates were washed three times and dried at 60°C for 30 min, then 200 μL of 0.1% (w/v) crystal violet solution was added to each well for staining. The stain retained by the biofilm was dissolved in 200 μL of 33% glacial acetic acid and the OD value was measured at 595 nm.

Measurement of the Metabolic Activity of Biofilm

The biofilm metabolic activity of the biofilm was detected using standard XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Aladdin, Shanghai, China) reduction assay.[24] XTT solution (1 mg/mL) was prepared in sterile distilled water and menadione solution (1 mmol/L, Aladdin, Shanghai, China) was dissolved in acetone. After the plate was washed three times with PBS, 54 μL of menadione/XTT mixed solution (menadione: XTT=1:12.5) was added to each well, and the reaction was carried out at 35°C for 2 h in the dark. The absorbance at 490 nm was measured.

Analysis of the Extracellular Polysaccharide (EPS)

EPS was extracted by previously reported methods.[25,26] Briefly, *C. albicans* suspensions (1%, v/v) were transferred to the fresh culture medium, and after treatment with cordycepin, the suspensions were centrifuged at 4, 000 ×g for 10 min to remove the cells. The EPS in the supernatant was then precipitated in 80% (v/v) cold ethanol and stored at 4°C overnight. The supernatant was removed after centrifugation at 8, 000 ×g for 20 min at 4°C. For polysaccharide precipitation, 1 mL of 0.5% phenol and 5 mL of sulfuric acid were added to each experimental group at the same rate. Afterward, the tubes were allowed to stand for 10 min and then the solution appeared red when maintained in a boiling water bath for 15 min. Changes in EPS could be quantified through the value of $\text{OD}_{490\text{nm}}$.

Observation of Biofilm by Confocal Laser Scanning Microscopy (CLSM)

C. albicans suspensions were added into a 6-well plate containing a sterilized coverslip and medium. Cordycepin was added at different time points. One group was that cordycepin was added at the beginning and incubated with *C. albicans* for 24 h. The other group was that cordycepin was added after 24 h and then incubated for 4 h. The coverslips were washed three times to remove unadhered microorganisms. Immediately after the excess water was aspirated, 5 μL of fluorescein isothiocyanate (FITC) Cona

(Sigma Aldrich, UK) was added and stained for 30 min at 4°C. Then 5 μL of propidium iodide (PI, Aladdin, Shanghai, China) solution was added at the same position and stained for 15 min at 4°C. At the same time, 20 μL of a mixed solution of fluorescein diacetate (FDA, Aladdin, Shanghai, China) and PI (FDA:PI = 1:1) was added to another set of coverslips and stained for 15 min at room temperature. CLSM (ZEISS, Germany) was used to observe the coverslip that has been placed on the slide.[22,27]

Cellular Surface Hydrophobicity (CSH) Assay

CSH analysis was based on water–hydrocarbon interface analysis as described by Stephen and Sardi et al.[28,29] The drug-treated fungal suspensions were washed twice and resuspended in PBS solution (denoted as A_0, OD_{600} = 0.4–0.6). One milliliter of toluene was added to 1.5 mL of the suspension. Thereafter, the mixture was vortexed for 2 min and allowed to stand for 10 min. Finally, the lower aqueous phase was taken with a syringe to determine the OD value (A_1) at 600 nm. Cells in RPMI-1640 medium without toluene were used as negative controls. The relative CSH was expressed as the percentage drop in optical density of the test suspension compared with the negative control.[30] The formula was:

$$\text{Relative CSH}(\%)=\frac{A_0-A_1}{A_1}\times 100\%$$

Acute Toxicity

All animal studies were approved by the Laboratory Animals Ethics Committee of Jiangnan University and carried out in compliance with national and international (Declaration of Helsinki) guidelines. For the acute toxicity test, the 30 KM mice (18–22g) purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Beijing, China) were separated into three groups as 500 mg/kg, 250 mg/kg, and PBS control group (n=10). The mice were fasted overnight and then administered. After that, the mice's weight, mental state, hair condition and death were daily observed for 14 days.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 statistical software. Data were expressed as mean ± standard deviation and analyzed by one-way analysis of variance (AVONA). A *p*-value below 0.05 was considered statistically significant.

Results

MICs of Cordycepin

Cordycepin was tested for its antifungal activity against *Candida* pathogens, and the MIC values of cordycepin are listed in Table 1. Cordycepin exhibited antifungal activity against the tested strains, and the antifungal activity of cordycepin against *C. albicans* was higher than that against *C. parapsilosis, C. glabrata*, and *C. tropicalis*. To further evaluate the inhibitory effect of cordycepin on *C. albicans*, fluconazole was used as a positive control. The MIC_{50} values of fluconazole against standard and clinically isolated *C. albicans* strains were ≤0.002 and 0.032 mg/mL, and the antifungal activity of fluconazole against *C. albicans* standard strains was consistent with the value determined in a previous study.[3] According to the breakpoints for in vitro broth dilution susceptibility testing of *C. albicans* in CLSI document M60 that sets the MIC_{50} of fluconazole against *C. albicans* as ≤0.002 mg/mL, the strain is susceptible to fluconazole. When MIC_{50} = 0.004 mg/mL, *C. albicans* is susceptible-dose dependent to fluconazole. When MIC_{50} ≥ 0.008 mg/mL, *C. albicans* is resistant to fluconazole.[31] Therefore, the clinical *C. albicans* isolate was resistant to fluconazole, but the standard strains were susceptible. For the next tests, the clinical isolate of *C. albicans* was selected.

Effects of Cordycepin on the Growth of *C. albicans*

The effects of cordycepin on the growth of *C. albicans* were tested by measuring the optical density of fungal culture at 600 nm in the presence or absence of cordycepin, as shown in Figure 1. In the absence of cordycepin,

Table 1 MIC Values of Cordycepin and Fluconazole

Drugs	Species	MIC_{50} (mg/mL)
Cordycepin	*C. albicans* BNCC 186382	0.062
	C. albicans ATCC 10231	0.062
	C. albicans CMCC 98001	0.031
	C. albicans	0.062
	C. tropicalis	0.125
	C. glabrata	0.125
	C. parapsilosis	0.25
	Species	MIC_{50} (mg/mL)
Fluconazole	*C. albicans* BNCC 186382	0.001
	C. albicans ATCC 10231	0.002
	C. albicans CMCC 98001	0.002
	C. albicans	0.032

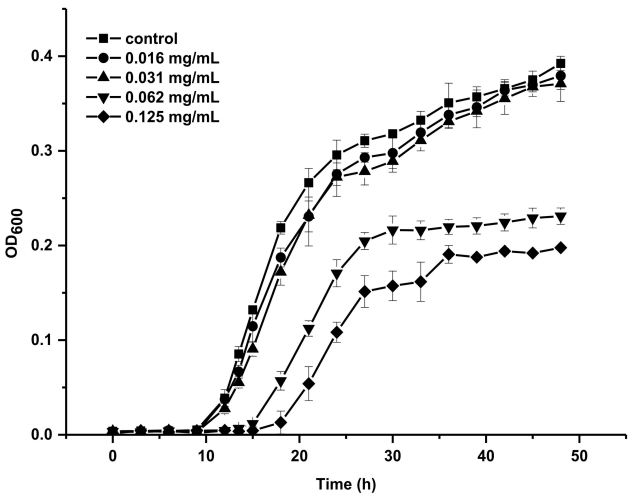

Figure 1 Growth curves of *C. albicans* in the absence or presence cordycepin.

C. albicans reached exponential phase within 9 h. At concentrations of 0.031 and 0.016 mg/mL, there were no significant effects on the growth of *C. albicans* compared with the control ($P > 0.05$). For concentrations of 0.062 and 0.0125 mg/mL, the growth of *C. albicans* was significantly inhibited ($P < 0.05$).

Effects of Different Materials on the Ability of *C. albicans* to Form Biofilms

Plastic, glass, and stainless steel materials are commonly used to make various medical devices that are used in hospitals. Pathogenic fungi can form biofilms and attach to devices including intravenous catheters, shunts, and stents, posing a potential source of infection for patients.[32–34] We tested various materials and found that *C. albicans* could form biofilms on plastic, glass and stainless steel materials with OD595 values of the resulting biofilms of 3.907 ± 0.111, 3.799 ± 0.166, and 0.741 ± 0.064, respectively. The ability to form biofilms on plastic polypropylene and stainless steel was equivalent ($P > 0.05$), with the two groups significantly different from the glass group ($P < 0.05$). This may reflect the higher surface roughness of plastics and stainless steel compared to glass, so that *C. albicans* can more easily be adsorbed on their surface.[35] *C. albicans* exhibited the strongest biofilm formation ability on polypropylene materials, suggesting that *C. albicans* is more likely to form biofilms on polypropylene materials, which will present a significant health risk. To further investigate biofilm formation, we used 96-well plates and test tubes made of polypropylene to cultivate strains for experiments.

Inhibitory Effects of Cordycepin on the Formation of *C. albicans* Biofilm

Biofilm formation was measured by crystal violet staining. Cordycepin showed significant inhibition on the biofilm formation of *C. albicans*, with a significant increase in inhibition with increase of cordycepin concentration (Figure 2A). The formation of *C. albicans* biofilm was reduced by 68.45% in the presence of 0.125 mg/mL of cordycepin compared with the control group, indicating effective inhibition of *C. albicans* biofilm formation at this concentration. For concentrations of 0.032 and 0.016 mg/mL, cordycepin had no effect on the growth of the strain but inhibited the formation of biofilm by 46.52% and 28.22%, respectively.

Eradicating Effects of Cordycepin on Pre-Formed Biofilms of *C. albicans*

The structure of mature biofilms is firm, stable, and not easily damaged, making it difficult for generic drugs to reduce or eliminate mature biofilms. We found that the ability of cordycepin to eradicate pre-formed biofilms of *C. albicans* was concentration-dependent, which meant that the higher the concentration, the stronger the scavenging effect became (Figure 2B). At a concentration of 0.5 mg/mL, the eradication percentage was 59.14%, significantly improved compared with that of 0.125 mg/mL ($P < 0.05$). The results indicate that high concentration of cordycepin can effectively eliminate pre-formed biofilms.

Effects of Cordycepin on the Formation and Eradication of Biofilm Observed by CLSM

CLSM was used to evaluate the effect of cordycepin on biofilm using FDA/PI fluorescent staining. The staining results revealed a much larger number of viable cells in the control group compared to the number of dead cells, with a thick and large biofilm (Figure 3A, B). After treatment with 0.032 mg/mL of cordycepin, the thickness of biofilm decreased significantly (Figure 3C and D). At a concentration of 0.125 mg/mL, the thickness of the biofilm and the total amount of cells were significantly reduced, with only a few cells remaining adhered to the cover glass (Figure 3E and F), indicating that cordycepin (0.125 mg/mL) effectively disrupts biofilms of *C. albicans*.

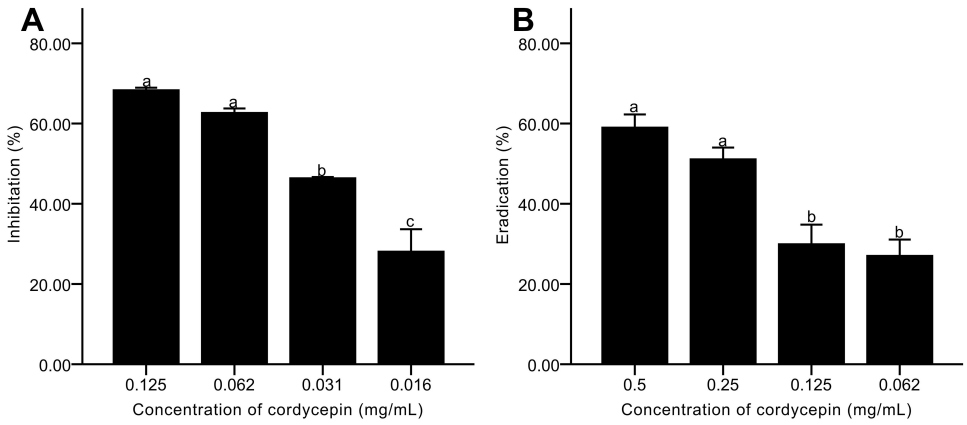

Figure 2 Effects of cordycepin on biofilms of *C. albicans*. (**A**) Inhibitory effects on biofilm formation; (**B**) Eradicating effects on mature biofilms. Error bars represent the standard deviations, and different letters represent statistical differences among bars (n = 3, P < 0.05).

After treatment of the pre-formed biofilms with 0.125 and 0.5 mg/mL of cordycepin, biofilm thickness and the ratio of living cells to dead cells were obviously decreased compared to those of the pre-formed biofilms that were not treated with cordycepin (Figure 4). With increasing concentration, the changes were more obvious. This indicates that a high concentration of cordycepin can get through the extracellular polymer to enter the biofilm, thus killing *C. albicans*. Figure 4 shows the partial eradication of mature biofilms with severe damage to biofilm structure.

Effects of Cordycepin on Metabolic Activity of *C. albicans* Biofilm

During metabolism, microbial biofilms secrete substances including polysaccharides, proteins, nucleic acids, and other substances that are essential for biofilm. XTT colorimetric method was used to evaluate the metabolic activity of *C. albicans* biofilm.[36] In the biofilm formation process, when the concentrations of cordycepin were at 0.062, 0.032, and 0.016 mg/mL, the metabolic activity of biofilm was less than 50% (Figure 5A). At a concentration of 0.125 mg/mL, there was greater inhibitory effect of cordycepin, with inhibition percentage of 63.11%. The results show that cordycepin inhibits the metabolic activity of *C. albicans* to decrease the ability of *C. albicans* to form biofilms.

After the biofilm is formed, it is difficult for antifungal drugs to act on microorganisms encapsulated by extracellular matrix. Mature biofilms are highly resistant to fluconazole, amphotericin B, and other drugs.[37] To assay the effect of cordycepin on the activity of mature biofilms of *C. albicans*, pre-formed biofilms were exposed to different concentrations of cordycepin. We observed that cordycepin had an obvious effect on mature biofilm of *C. albicans* (Figure 5B). At concentrations of 0.125 and 0.062 mg/mL, the reduction percentages were only 23.67% and 12.17%. However, treatment with 0.5 mg/mL cordycepin concentration resulted in a reduction percentage of 56.08%. As the concentration increased, more cordycepin likely is able to enter the mature biofilms, leading to a significant decrease in metabolic activity.

Effects of Cordycepin on EPS of Biofilm

EPS was measured using a phenol-sulfuric acid method, and the results are shown in Figure 6. As the concentration of cordycepin increased, the secretion of EPS decreased steadily (Figure 6A). Addition of 0.125 mg/mL of cordycepin resulted in an EPS inhibition percentage of 60.52%. At lower concentrations, inhibition was less than 50%, indicating that cordycepin can more effectively reduce EPS secretion in biofilm formation at high concentrations.

After treating the pre-formed biofilms with 0.5 and 0.25 mg/mL of cordycepin, the EPS reduction percentages were 53.67% and 36.25%, respectively, and there was a significant difference in EPS secretion ($P < 0.05$) (Figure 6B). At concentrations of 0.125 and 0.062 mg/mL, the EPS reduction percentage was 20.04% and 10.44%. The results showed that cordycepin could significantly decrease EPS secretion in pre-formed biofilms, limiting the protective effect of extracellular polysaccharides to maintain biofilm.[38]

Effects of Cordycepin on EPS of Biofilm Observed by CLSM

In order to further understand the three-dimensional structural changes of biofilms, CLSM was used to observe the

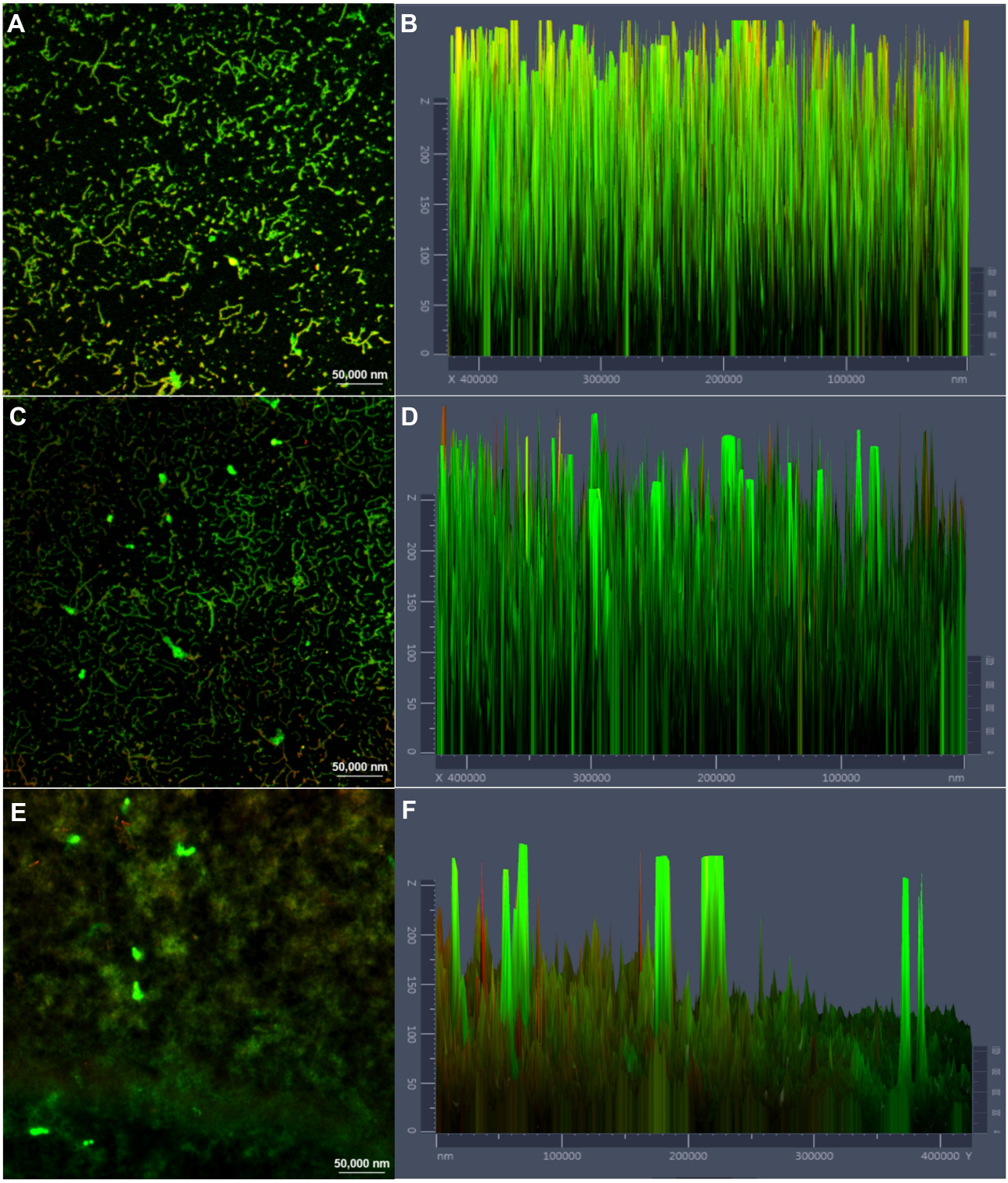

Figure 3 CLSM images of *C. albicans* biofilm treated with different concentrations of cordycepin. (**A**), (**C**) and (**E**) show the effects of 0, 0.031, and 0.125 mg/mL of cordycepin on the formation of biofilms and live/dead cells ratio. (**B**), (**D**) and (**F**) show the fluorescence intensities of live (green)/dead (red) cells in biofilms treated with 0, 0.031, and 0.125 mg/mL of cordycepin, respectively. Viable cells appear green due to FDA staining, and cells with damaged membranes appear red due to PI staining.

distributions of EPS and dead cells.[22] Staining results revealed that the polysaccharides in the untreated biofilm were densely distributed and adsorbed on the coverslip in a lump (Figure 7A). After treatment with 0.032 mg/mL of cordycepin, there was decreased fluorescence intensity of EPS (Figure 7C). The EPS treated with 0.125 mg/mL of

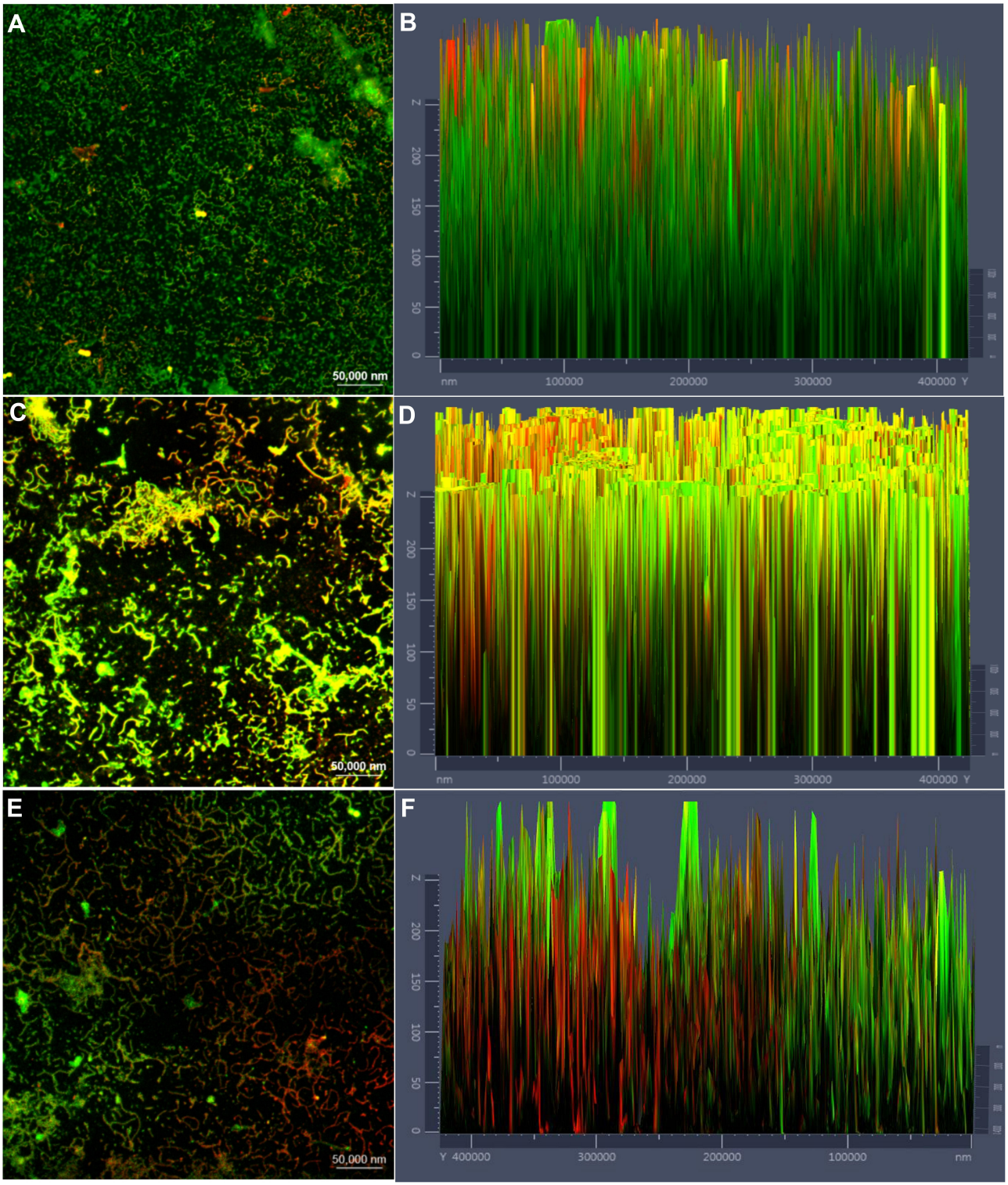

Figure 4 Changes of mature biofilms and live/dead cells before and after cordycepin treatment. (**A**), (**C**) and (**E**) show the effects of 0, 0.125, and 0.5 mg/mL of cordycepin on the eradication of mature biofilms and live/dead cells. (**B**), (**D**) and (**F**) show the fluorescence intensities of live (green)/dead (red) cells in biofilms treated with 0, 0.125, and 0.5 mg/mL of cordycepin, respectively. Viable cells appear green due to FDA staining, and cells with damaged membranes appear red due to PI staining.

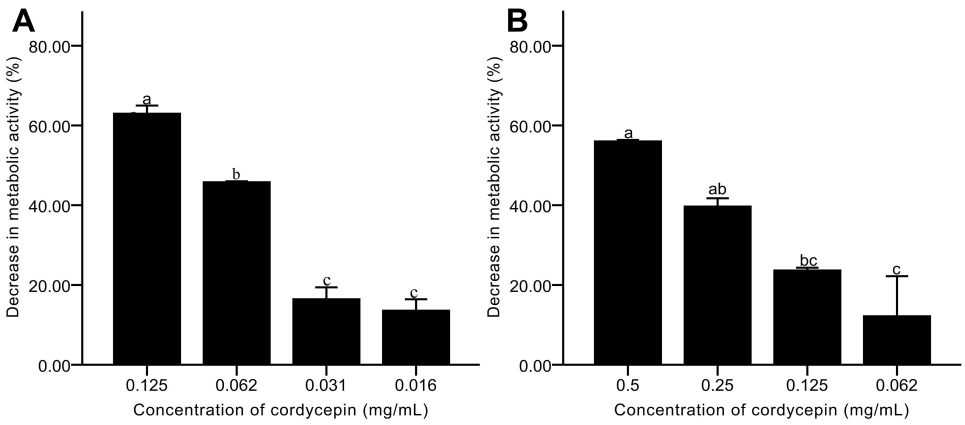

Figure 5 Effects of cordycepin on the metabolic activity of *C. albicans* biofilm. (**A**) Effects on biofilm formation; (**B**) Effects on mature biofilms. Error bars represent the standard deviations, and different letters represent statistical differences among bars (n = 3, P < 0.05).

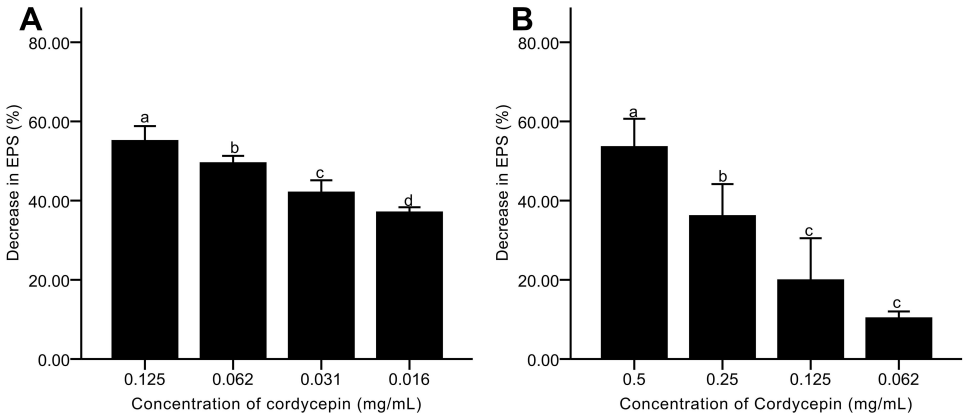

Figure 6 Effects of cordycepin on EPS secretion of *C. albicans* biofilm. (**A**) Effects on EPS secretion in biofilm formation; (**B**) Effects on EPS secretion in mature biofilms. Error bars represent the standard deviations, and different letters represent statistical differences among bars (n =3, P < 0.05).

cordycepin exhibited a loose distribution, with obvious differences from treatment with 0.032 mg/mL of cordycepin (Figure 7E), indicating that cordycepin significantly inhibited the formation of *C. albicans* biofilm.

The effect of cordycepin on the EPS of pre-formed biofilms exhibited the following trend. As the cordycepin concentration increased, the fluorescence intensity of the polysaccharide became weaker, the distribution became sparser, and the number of dead cells increased (Figure 7B, D and F). In the control group without cordycepin treatment, the EPS secreted by *C. albicans* showed strong fluorescence intensity, and the cells were visibly embedded (Figure 7B). After treatment with 0.125 mg/mL of cordycepin, the content of EPS decreased. Its distribution was not as dense as the control group, and cells were more easily seen (Figure 7D). At a concentration of 0.5 mg/mL, the polysaccharides were loosely distributed with little aggregated into clusters, and significantly increased amount of dead cells (Figure 7F). Overall, these results indicate that cordycepin can reduce the secretion of EPS, destroy biofilm structure, and affect cell-cell and cell–environment interactions to disrupt biofilms.[39–41]

Effect of Cordycepin on CSH of *C. albicans* Biofilm

Adhesion is an important stage in biofilm formation of *C. albicans*, and CSH is positively correlated with *C. albicans* adhesion.[42–44] We first investigated the effect of cordycepin on CSH of *C. albicans* during the formation of biofilm. The control group without cordycepin had a relative CSH value of 51.02% after 24 h of culture, indicating a high hydrophobic capacity. With the addition of cordycepin at an initial stage, the relative CSH of the experimental group was reduced. A concentration of 0.125 mg/mL cordycepin had the biggest effect, reducing relative CSH to 21.53% (Figure 8A). We next tested the effect of cordycepin after biofilm formation. To do this, we

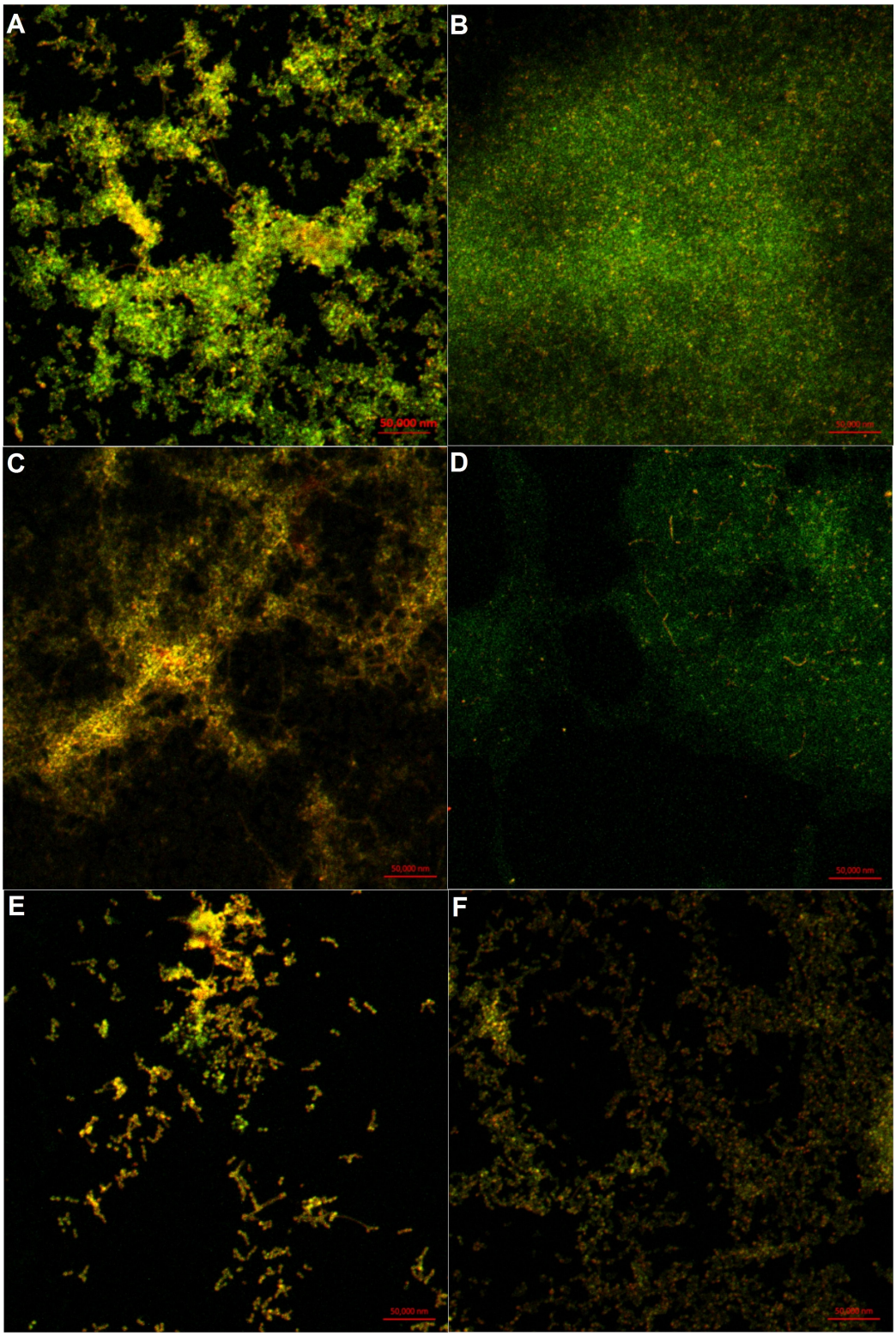

Figure 7 Distribution of polysaccharides and dead cells in cordycepin-treated biofilms. (**A**), (**C**) and (**E**) show the distribution of EPS (green) and dead cells (red) in biofilms treated with 0, 0.031, and 0.125 mg/mL of cordycepin. (**B**), (**D**) and (**F**) show the distribution of EPS (green) and dead cells (red) in mature biofilms treated with 0, 0.125, and 0.5 mg/mL of cordycepin.

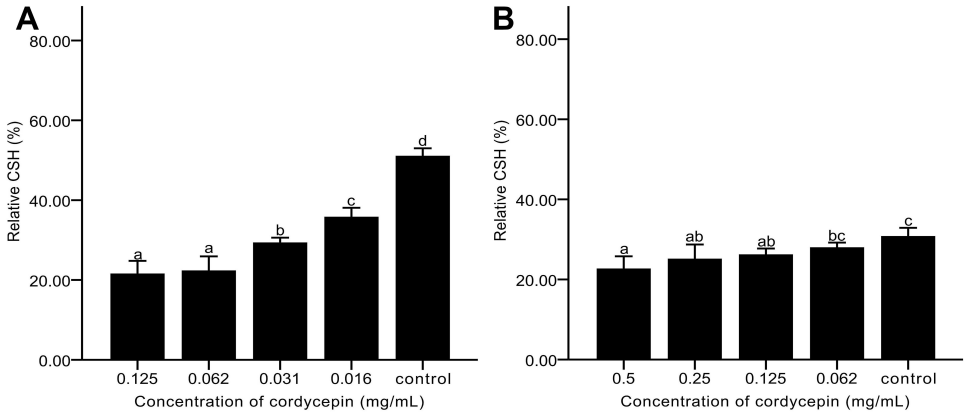

Figure 8 Effects of cordycepin on CSH of *C. albicans* biofilm. (**A**) Addition of cordycepin before biofilm formation; (**B**) Addition of cordycepin after biofilm formation. Error bars represent the standard deviations, and different letters represent statistical differences among bars (n = 3, P < 0.05).

cultured *C. albicans* for 24 h, and then added different concentrations of cordycepin. The results showed that the hydrophobicity of the control group without treatment decreased, which may be related to the culture time (Figure 8B).[45] However, the relative CSH of this group was higher than the group with the addition of cordycepin. These results indicate that cordycepin can affect the adhesion of *C. albicans* and reduce the likelihood of biofilm formation. The addition of cordycepin before the formation of biofilm had the biggest effect on adhesion.

Acute Toxicity

To fully evaluate the potential to use cordycepin, the acute toxicity test was performed to determine the LD_{50} (50% of lethal dose) in mice. After sacrificing, the size and appearance of the organs of the mice in the experimental group and the control group were normal, with no obvious lesions such as exudation, hyperplasia, edema, or atrophy in tissues and organs. Therefore, the LD_{50} of cordycepin was determined to be higher than 500 mg/kg for mice.

Discussion

The emergence of resistance to commercial drugs and the toxicity of drugs have limited the effectiveness of current treatment options.[4] The resistance of pathogens to conventional drugs has multiple mechanisms, such as reducing membrane permeability, encoding multidrug efflux pumps, forming biofilms, and inactivating cell membrane receptors.[46–49] Therefore, natural, safe and effective antibiotic substitutes are what we have been pursuing. Many natural compounds are reported to have antimicrobial activity against pathogenic microorganisms, including essential oils, curcumin and anthocyanins.[50–52] Previous studies have shown that essential oils from leaves of *Paramignya trimera, Limnocitrus littoralis* and *Leoheo domatiophorus* inhibited the growth of *C. albicans* with MICs of 16%, 16%, and 4% (v/v), respectively.[53,54] In this study, we determined the MIC_{50} of cordycepin against *C. albicans* standard strains by broth dilution method. The MIC_{50} of cordycepin was ranging from 0.031 to 0.062 mg/mL, which was higher than that of fluconazole (-≤0.002 mg/mL), indicating that more cordycepin is needed to achieve the same effect. However, there is a possibility for greater efficacy using cordycepin in combination with other antibiotics. For example, the MIC of eugenol against *C. albicans* (ATCC 90028) was 625 μg/mL, whereas in combination with amphotericin B (0.05 μg/mL), the MIC of eugenol dropped to 156 μg/mL.[55] Therefore, the application of cordycepin in combination with other antibiotics will be explored in our future work.

Although natural products and their derivatives have antimicrobial potential, they are still limited as anti-biofilm agents.[56] To find effective agents to prevent and control biofilm, researchers have started to evaluate small molecules of secondary metabolites that are produced by organisms as a protective response to the external environment.[57,58] For microorganisms, the secretion of secondary metabolites is a means to compete with other species for resources in the living environments.[59] After millions of years of evolution and natural selection, microorganisms can not only retain a high degree of selectivity and potency, but also the metabolites they produce sometimes affect the cell envelope, which is lacking in synthetic drugs.[60] Walkmycin C is produced by *Streptomyces* sp. strain MK632-100F11 and causes a reduction in *Streptococcus mutans* biofilms by inhibiting the

autophosphorylation of histidine kinases VicK and CiaH in a two-component system.[61]

In this study, we found that cordycepin as a nucleoside analogue and a secondary metabolite of *C. militaris* exhibited antifungal activity against *Candida* species but also effectively inhibited the formation of *C. albicans* biofilm and eradicated mature biofilms. In addition, cordycepin could inhibit the formation of biofilm at a low concentration without affecting significantly cell growth. Another natural nucleoside component, sinefungin can decrease the formation of *Streptococcus pneumoniae* biofilm by 15% and 53% at concentrations of 10 and 50 μg/mL.[62] Pierce et al found that tunicamycin, a nucleoside antibiotic, can inhibit the formation of *C. albicans* biofilm, but has little effect on mature biofilms.[37]

However, the specific anti-biofilm mechanism of cordycepin has not yet been described. In general, anti-biofilm agents may prevent biofilm adhesion, anchoring and matrix production by targeting cellular processes or interfering with quorum sensing.[20] Tapia-Rodriguez reported that biofilm inhibition of carvacrol could be related to the interruption of quorum sensing, potentially achieved by inhibition of the production of pyocyanin and violacein in *Pseudomonas aeruginosa* and *Pseudomonas purple*.[63] Quercetin inhibits the formation of *Listeria monocytogenes* biofilm by reducing initial attachment and protein accumulation in the matrix.[64] In addition, the inhibition of *C. albicans* biofilm by tunicamycin can be explained by N-linked glycosylation defects, leading to changes in the properties of the cell wall and surface mannoproteins as well as CSH.[37] Similarly, we observed that cordycepin significantly decreased metabolic activity, relative CSH, and EPS secretion in a concentration-dependent manner, and these findings were consistent with our CLSM observations.

Mature biofilms are difficult to be eradicated by clinical drugs, such as amphotericin B, fluconazole, flucytosine and itraconazole, which are much less active against *C. albicans* biofilm than planktonic cells.[65] In this study, cordycepin exhibited a clear eradicating effect on pre-formed biofilms, with an eradication percentage of 59.14% after treatment with 0.5 mg/mL cordycepin for 4 h. The results show that cordycepin effectively reduced the secretion of the extracellular matrix, affected the structure of the biofilm, and was able to act on cells wrapped by the extracellular matrix.

Overall, our results indicate that cordycepin, a natural nucleoside component, may be applied as an antifungal and anti-biofilm agent against *C. albicans*.

Conclusion

The results showed that the MIC_{50} of cordycepin against *C. albicans* was 0. 063 mg/mL. At a concentration of 0.125 mg/mL, cordycepin effectively inhibited the formation of biofilms, with an inhibition percentage of 68.45%. At concentrations less than or equal to 0.032 mg/mL, cordycepin had no effect on strain growth but inhibited the formation of biofilm. In addition, a high concentration of cordycepin showed a significant eradicating effect on mature biofilms of *C. albicans*. The inhibiting and eradicating effects of cordycepin were achieved by decreasing the metabolic activity, the living cells, and the hydrophobicity, and by damaging extracellular polysaccharide of biofilm. Acute toxicity was assessed and revealed that the LD_{50} of cordycepin was higher than 500 mg/kg for mice. These results indicate that cordycepin provides a new alternative to combat and eliminate fungal biofilms, which is beneficial to design new drugs and reduce drug resistance.

Ethical Approval

This study has been approved by the Ethics Committee of Wuxi No. 2 People's Hospital 20171115 and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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Disclosure

The authors report no conflicts of interest in this work.

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