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Association of different *Candida* species with catheter-related candidemia, and the potential antifungal treatments against their adhesion properties and biofilm-forming capabilities

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Abstract

Background: To compare the adhesion properties and biofilm-forming capabilities of 27 *Candida* isolates obtained from catheter-related candidemia patients and to evaluate the inhibitory effects of antifungal agents on different *Candida* species.

Material and Methods: Seven *C. albicans*, six *C. parapsilosis*, five *C. guilliermondii*, five *C. tropicalis*, and four *C. glabrata* clinical isolates were investigated. We quantified the adherence of these *Candida* species by flow cytometric method and evaluated the formation of biofilms by XTT reduction and crystal violet methods. Actions of micafungin (MF), fluconazole (FZ), and N-acetylcysteine (NAC) on the adhesion and biofilm formation of different *Candida* species were determined.

Results: Non-*albicans Candida* species were demonstrated to have stronger adhesion abilities compared with *C. albicans*. The biofilm-forming capabilities of different *Candida* species were varied considerably, and the degree of biofilm formation might be affected by different assay approaches. Interestingly, *C. parapsilosis* displayed the highest biofilm formation abilities, while *C. glabrata* exhibited the lowest total biomass and metabolic activity. Furthermore, the inhibitory activities of MF, FZ, and NAC on fungal adhesion and biofilm formation were evaluated, and the results indicated that MF could reduce the adhesion ability and biofilm metabolism more significantly ($p < 0.05$), and its antifungal activity was elevated in a dose-dependent manner.

Conclusion: Non-*albicans Candida* species, especially *C. guilliermondii*, *C. tropicalis*, and *C. parapsilosis*, exhibited higher adhesion ability in catheter-related candidemia patients. However, these *Candida* species had varied biofilm-forming capabilities. MF tended to have stronger inhibitory effects against both adhesion and biofilm formation of different *Candida* species.

KEYWORDS
adhesion, antifungal agents, biofilms, non-*albican Candida*

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1 | INTRODUCTION

Candida species are presently ranked as a top cause of bloodstream infections (BSIs) with high mortality and morbidity.[1-6] Candidemia is highly associated with the formation of biofilms on central venous catheters (CVCs), which can provide adhesion surfaces for *Candida* species to colonize and form biofilms, thus contributing to BSIs.[4,7-9]

An epidemiological shift has been reported in the past decades. Although *Candida albicans* (*C. albicans*) remains dominant in bloodstream, non-*albicans Candida* species (e.g., *C. parapsilosis*, *C. tropicals*, and *C. glabrata*) have been gradually recognized.[10-13] As one of the major non-*albicans Candida* species, *C. parapsilosis* was found in patients with central line-associated candidemia, and CVC-related candidemia was more likely to be biofilm positive.[14-17]

Biofilm-forming *Candida* species are more resistant to antimicrobial agents [18-22], hence, the development of *Candida* biofilms on medical implant devices can trigger an intractable clinical problem. Although most studies have focused on the biofilms formed by *C. albicans*,[18,23-25] there is little information available on non-*albicans Candida* biofilms. Recently, Pannanusorn *et al* [26] found that biofilms, as a major virulence determinant, could be more beneficial for non-*albicans Candida* species rather than *C. albicans*. Given that non-*albicans Candida* are resistant to fluconazole, the antifungal activity of other antifungal agents during biofilm growth remains largely unknown.

This research aimed to compare the adhesion properties, biofilm formation, and sensitivities of 27 clinical *Candida* isolates toward antifungal agents and to evaluate the adhesion abilities and biofilm-forming capabilities of different *Candida* species.

2 | MATERIALS AND METHODS

2.1 | Definitions and microbiologic methods

Patients with catheter-related bloodstream infections (CRBSIs) were defined based on the current recommendations.[27-31] (i) Blood cultures from two body sites were positive for *Candida* species, along with clinical manifestations of yeast infection. (ii) Blood cultures and the catheter tip culture should be performed simultaneously, and both sites should be colonized with the same *Candida* species or the quantitative blood culture of the CVC tip sample showed a ≥5-fold greater CFU counts than the concurrent peripheral vein culture. All clinical isolates were cultured in accordance with the standard microbiologic method. Approval for the protocol was obtained from the local Ethics Committee at Renmin Hospital of Wuhan University (RHWU).

2.2 | Strains

Twenty-seven *Candida* isolates, including seven *C. albicans*, six *C. parapsilosis*, five *C. guilliermondii*, five *C. tropicalis*, and four *C. glabrata*, causing CRBSIs were evaluated for their adhesion properties, biofilm-forming capabilities, and antifungal susceptibilities. All *Candida* isolates were obtained from ICU patients in Renmin Hospital of Wuhan University (Table S1). *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028 were used as control strains.

All CRBSI samples were assessed using IVD MALDI Biotyper mass spectrometry (Bruker, Germany). Considering the purity and viability of the microorganisms, all *Candida* isolates were subcultured twice on Sabouraud Dextrose Broth (SDB; Hope Biotechnology, Qingdao) at 37°C for 24 h under 190 *g* rotation prior to each experiment.

2.3 | Antifungal susceptibility

Based on the identification results, antifungal susceptibility experiment was initiated using a commercial broth microdilution method (Sensititre YeastOne plate of Trek Diagnostic system, Thermo Fisher, CN15009). The minimal inhibitory concentrations (MICs) of all isolates were evaluated against fluconazole (FZ), amphotericin B (AMB), caspofungin (CAS), micafungin (MF), voriconazole (VOR), and posaconazole (PZ). Based on the recommendations of antifungal susceptibility testing documents M27 and M60 from Clinical and Laboratory Standards Institute (CLSI; M60, 1st edition),[32] the results were classified as susceptible, intermediate, susceptible-dose-dependent, and resistant.

2.4 | Adhesion method

Different *Candida* strains were prepared in SDB overnight at 37°C and 180 rpm. The concentration of the suspension was standardized to 2 MCF (≈4 × 10^6 cfu/ml); DensiCHEK™ PLUS, Thermo Fisher). After centrifuging at 3,164 *g* for 3 min and washing twice with phosphate buffer saline (PBS), the yeast cells were harvested and their adhesion ability was evaluated using flow cytometry (FACSVerseTM, BD Biosciences, US) method.[33] Briefly, yeast cell suspensions were mixed with green fluorescent polystyrene microspheres (1.0 μm; yellow-green 505/515, F8823, Molecular Probes, Thermo Fisher) at a final concentration of 2 × 10^8 microspheres/ml. Subsequently, the mixture was incubated at ambient temperature with agitation for 30 min. Single yeast cell and microspheres were set as controls. The suspensions were vortexed after incubation, and the flow cytometric results with 50,000 events were collected and analyzed. The following two parameters were selected to clarify the results: P1 and P2 stand for the percentages of yeast cells unattached and attached with microspheres, respectively. All results were derived from at least three independent experiments.

2.5 | Biofilm formation assay

Candida strains were recovered at 37°C for 1 h, then vortex for 5–10 s. All samples were grown in SDB at 37°C, 180 rpm for 24 h.

Cells were harvested followed by centrifugation (3,000 rpm, 3 min) and washed with PBS, and the turbidity of each suspension was adjusted to the equivalent of 1 MCF (≈2 × 10^6 cfu/ml) with SDB. Following, 100 μL aliquots of yeast cells suspensions were placed into the wells of a 96-well polystyrene microplates and incubated for 24 h at 37℃ for biofilm formation.[34-36]

To qualify the biofilms in each well, two distinct methods were used to evaluate biofilm formation in terms of both quantitative estimation and metabolic activity.[34] For XTT assay, after incubation for 24 h, 0.5 mg/mL of XTT (CAS number: 111072-31-2, Sigma) and 1 mmol/L of methylnaphthalene (CAS number: 58-27-5, Sigma) were prepared and mixed. Approximately 100 μL of the mixture was added into the 96-well plate and incubated for 2 h at 37℃. The absorbance values were read on a Microplate Photometer (Thermo Scientific™ Multiskan™ FC) at OD 450 nm. For CV assay, after incubation, 100 μL of formaldehyde solution (10%) was added to fix the cell suspensions for 2 min at room temperature. Then, 100 μL of 20 mg/ml CV solution (CAS number: 548-62-9, Sigma) was added and incubated for 30 min. Decolorization was performed by using 100 μl of 95% ethanol. The absorbance values were read on the Microplate Photometer at OD 630 nm. Two standard strains (*C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028) were employed as controls. All results were derived from at least three independent experiments.

2.6 | Antifungal activity assay

To demonstrate whether antifungal agents can exhibit inhibitory effects on adhesion and biofilm formation, MF, FZ, and NAC were added to the yeast suspensions at different concentrations, separately (Table 1). The lowest concentration was determined according to the antifungal susceptibility testing results (Table S2). The highest concentration of MF was determined as 5.0 μg/ml and FZ as 256 μg/ml ~512 μg/ml according to previous literature.[37-39] NAC was prepared at the concentrations of 10 mg/ml and 50 mg/ml.[40,41] Untreated fungal suspensions and medium alone were set as positive and negative controls, respectively. Non-adhered cells were discarded after incubation for 24 h at 37°C, and the production of biofilms in presence of MF, FZ, and NAC was measured by XTT and CV methods as described previously. The formula CFU = R1 × P2 × 5000 was used to evaluate the inhibitory effects of these antifungal agents on fungal adhesion.

2.7 | Statistical analysis

Statistical tests were performed using GraphPad Prism ver. 6.0 for Windows. For the comparison of *C. glabrata* adhesion and biofilm measures at 24 h in the presence or absence of antifungal agents, Kruskal-Wallis test was used since its distribution pattern was not asymmetric. Meanwhile, for the comparison of other *Candida* species, ordinary one-way ANOVA test was used. In addition, multiple comparison tests were carried out by Holm-Sidak method. Difference between two species was deemed significant if the *p*-value was 0.05 or lower.
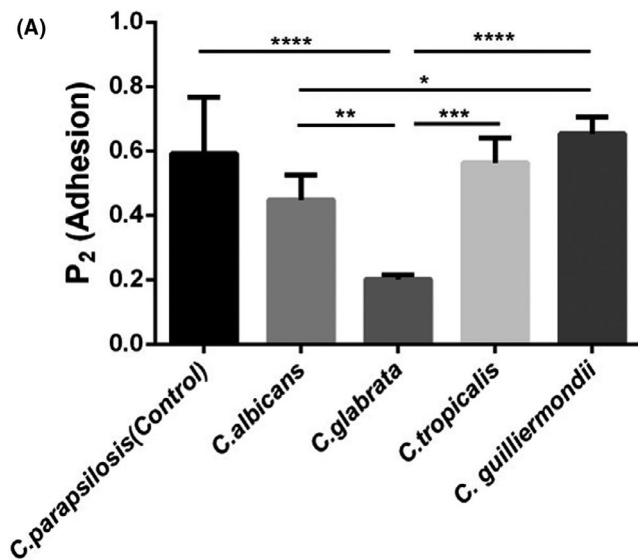
3 | RESULTS

3.1 | Microbiological susceptibilities of different *Candida* species

The antifungal susceptibilities of 27 clinical isolates recovered from CRBSIs, including seven *C. albicans*, six *C. parapsilosis*, five *C. tropicalis*, five *C. guilliermondii*, and four *C. glabrata*, were examined (Table S2). According to the established MIC breakpoints, all tested *C. albicans* isolates were sensitive to antifungal agents. For *C. glabrata*, three isolates were resistant, and one was intermediate to FZ; two isolates were resistant, and one was intermediate to VOR; two isolates were resistant to PZ; and none was resistant to AMB, CAS, and MF. With regard to *C. parapsilosis*, except that one isolate was resistant to FZ, others were susceptible to antifungal agents. All *C. tropicalis* isolates were susceptible to AMB, CAS, MF, and PZ, while four were resistant to VOR and the remaining one was resistant to FZ. All *C. guillermondii* isolates were resistant to both FZ and VOR, whereas no resistance was observed for AMB, CAS, MF, and PZ.

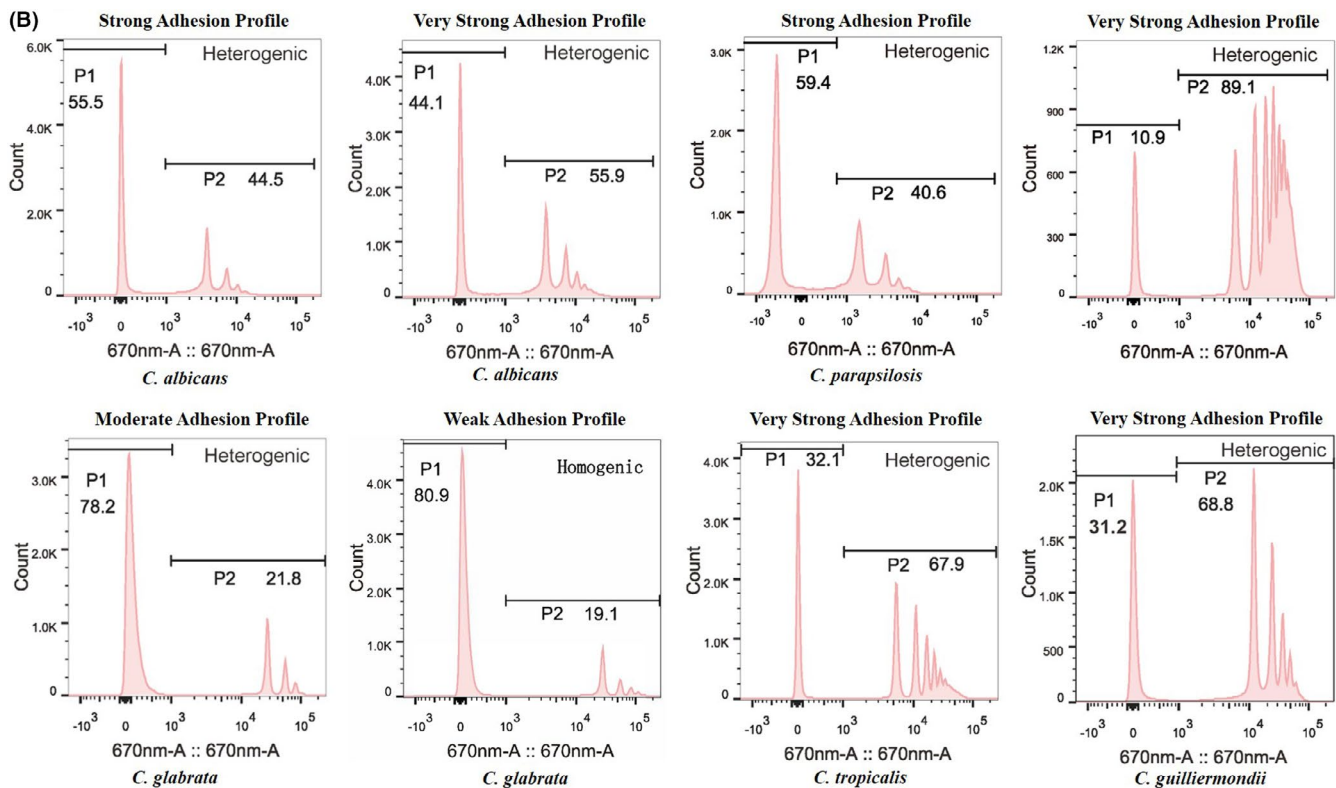### 3.2 | Adhesion profiles of different *Candida* species

The adhesion abilities of *Candida* species were evaluated by flow cytometry assay, as shown in Figure 1A and Table 2, and we observed an obvious difference among the adhesion percentages of different *Candida* species. Notably, *C. guilliermondii* (0.6540 ± 0.05154), *C. parapsilosis* (0.5919 ± 0.1749), and *C. tropicalis* (0.5636 ± 0.07692) exhibited much stronger adhesion abilities compared to *C. albicans* (0.4484 ± 0.07700) and *C. glabrata* (0.2023 ± 0.01284). To reduce

TABLE 1 Concentrations of micafungin (MF), fluconazole (FZ), and N-acetylcysteine (NAC) to different *Candida* species

Species	MF-mic (μg/ml)	MF-h (μg/ml)	FZ-mic (μg/ml)	FZ-h (μg/ml)	NAC-mic (mg/ml)	NAC-h (mg/ml)
C. albicans	0.03	5.0	2.0	256	10	50
C. glabrata	0.008	5.0	128	512	10	50
C. parapsilosis	0.5	5.0	2.0	256	10	50
C. tropicalis	0.03	5.0	256	512	10	50
C. guilliermondii	0.25	5.0	256	512	10	50

Abbreviations: H, high concentration; MIC, minimal inhibitory concentration.

FIGURE 1 (A) Adhesion of different *Candida* species were measured by flow cytometric assay. Data are represented by the mean values of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, versus controls. (B) Representation of *Candida* adhesion profiles. The isolates were characterized by weak, moderate, strong, and very strong adhesion patterns. Homogeneous distribution pattern means that yeast cells are attached to an equal amount of microspheres, while heterogeneous distribution pattern indicates that multiple microspheres are bound to a single yeast cell

the variability of adherent with microspheres (P2) between distinct *Candida* isolates and better evaluate the adhesion strength of each isolate, four adhesion profiles were established (Table S3 and Figure 1B). Weak adhesion isolates showed adhesion percentage (P2) ranging from 1% to 20%, and the strains with this adhesion pattern exhibited a homogeneous profile, implying that a single yeast cell is bound to each microsphere. Moderate adhesion isolates had adhesion percentage ranging between 21% and 30%, and the strains in this profile also demonstrated a homogeneous profile. Strongly adhering isolates had adhesion percentage between 31% and 50%, and the strains with this pattern exhibited a heterogeneous adhesion pattern, indicating that a single yeast cell is bound to more than one microspheres. Very strongly adhering isolates had adhesion percentage more than 50%, and the strains with this pattern also exhibited a heterogeneous adhesion pattern. Based on these adhesion patterns, we classified all the clinical isolates, and the results are summarized in Table 2. Among the tested *C. albicans* strains, 71.4% displayed a strong adhesion profile, while the remaining 28.6% exhibited a very strong profile. The majority of *C. glabrata* strains demonstrated a weak adhesion profile, except for *Cgl* 1. Almost all *C. parapsilosis* isolates displayed a very strong profile, and only one strain (*Cpa* 5) with strong adhesion. The most heterogeneous species were *C. guilliermondii* and *C. tropicalis*, all of which displayed a very strong adhesion profile.

Based on the adhesion results shown in Figure 1 and Table 2, we found that *the non-albicans* species, such as *C. guilliermondii*,

FIGURE 1 (Continued)

TABLE 2 Characterization of adhesion profile and biofilm formation of different *Candida* species

| Species | Strain | Adhesion | | | Biofilm | |
		% of cells with adherent microspheres (p2)	Distribution pattern	Adhesion profile	XTT (24H)	CV (24H)
C. albicans	Standard	37.7	Heterogenic	Strong	1.102	0.626
C. albicans	1	44.5	Heterogenic	Strong	1.025	0.378
C. albicans	3	44.5	Heterogenic	Strong	1.041	0.292
C. albicans	5	44.4	Heterogenic	Strong	1.062	0.363
C. albicans	6	55.9	Heterogenic	Very strong	1.089	0.441
C. albicans	11	38.1	Heterogenic	Strong	0.928	0.256
C. albicans	14	56.5	Heterogenic	Very strong	1.050	0.382
C. albicans	15	37.1	Heterogenic	Strong	1.054	0.536
C. glabrata	1	21.2	Homogenic	Moderate	0.942	0.271
C. glabrata	2	20.3	Homogenic	Weak	0.869	0.271
C. glabrata	3	20.9	Homogenic	Weak	0.708	0.333
C. glabrata	4	18.4	Homogenic	Weak	1.092	0.276
C. parapsilosis	Standard	48.3	Heterogenic	Strong	1.368	0.355
C. parapsilosis	5	40.6	Heterogenic	Strong	1.353	0.233
C. parapsilosis	6	77.6	Heterogenic	Very strong	1.412	0.280
C. parapsilosis	7	89.1	Heterogenic	Very strong	1.029	0.944
C. parapsilosis	8	51.8	Heterogenic	Very strong	1.181	0.200
C. parapsilosis	9	50.5	Heterogenic	Very strong	1.251	0.228
C. parapsilosis	10	56.4	Heterogenic	Very strong	1.031	0.936
C. tropicalis	3	52.4	Heterogenic	Very strong	1.063	0.255
C. tropicalis	4	60.6	Heterogenic	Very strong	1.133	0.289
C. tropicalis	5	50.4	Heterogenic	Very strong	1.132	0.303
C. tropicalis	6	50.5	Homogenic	Very strong	1.022	0.295
C. tropicalis	7	67.9	Heterogenic	Very strong	1.000	0.304
C. guilliermondii	1	71.9	Heterogenic	Very strong	1.151	0.405
C. guilliermondii	2	68.8	Heterogenic	Very strong	1.224	0.363
C. guilliermondii	3	64.3	Heterogenic	Very strong	1.109	0.389
C. guilliermondii	4	63.5	Heterogenic	Very strong	1.113	0.343
C. guilliermondii	6	58.5	Heterogenic	Very strong	1.135	0.375

Note: The adhesion pattern and adhesion profile was established. Biofilm formation at 24 h was evaluated by XTT and CV methods. Results were performed at least 3 independent experiments. P2: the percentage of yeast cells attached with adherent microspheres. Homogenic: a single microsphere was attached to each yeast cell. Heterogenic: more than a single microsphere was attached to each yeast cell.

C. parapsilosis, and *C. tropicalis*, had very strong adhesion pattern, and there were no significant differences among them. *C. albicans* showed moderate adhesion ability, which was lower than *C. guilliermondii* ($p < 0.05$) and higher than *C. glabrata* ($p < 0.01$). Besides, *C. glabrata* exhibited the lowest adhesion ability and was significantly different compared to *C. guilliermondii* ($p < 0.0001$), *C. parapsilosis* ($p < 0.0001$), *C. tropicalis* ($p < 0.001$), and *C. albicans* ($p < 0.01$).

3.3 | Biofilm formation of different *Candida* species

The biofilms of different *Candida* species formed on polystyrene well plates were measured by both XTT and CV methods.

XTT assay was conducted to examine the metabolic activity of the biofilms at 24 h, and the results (Figure 2A) indicated that *C. guilliermondii* and *C. parapsilosis* had the highest metabolic activities. Specifically, *C. guilliermondii* showed higher XTT reduction values than *C. albicans* ($p < 0.0001$), *C. glabrata* ($p < 0.0001$), and *C. tropicalis* ($p < 0.001$). Similarly, *C. parapsilosis* also exhibited higher XTT reduction values compared to *C. albicans* ($p < 0.001$), *C. glabrata* ($p < 0.0001$), and *C. tropicalis* ($p < 0.01$). However, there was no remarkable difference in XTT reduction values between *C. guilliermondii* and *C. parapsilosis*. In addition, CV assay was performed to measure the total biomass of the biofilms at 24 h, and the results (Figure 2B) demonstrated that *C. parapsilosis* produced a higher volume of biofilms compared to *C. glabrata* and
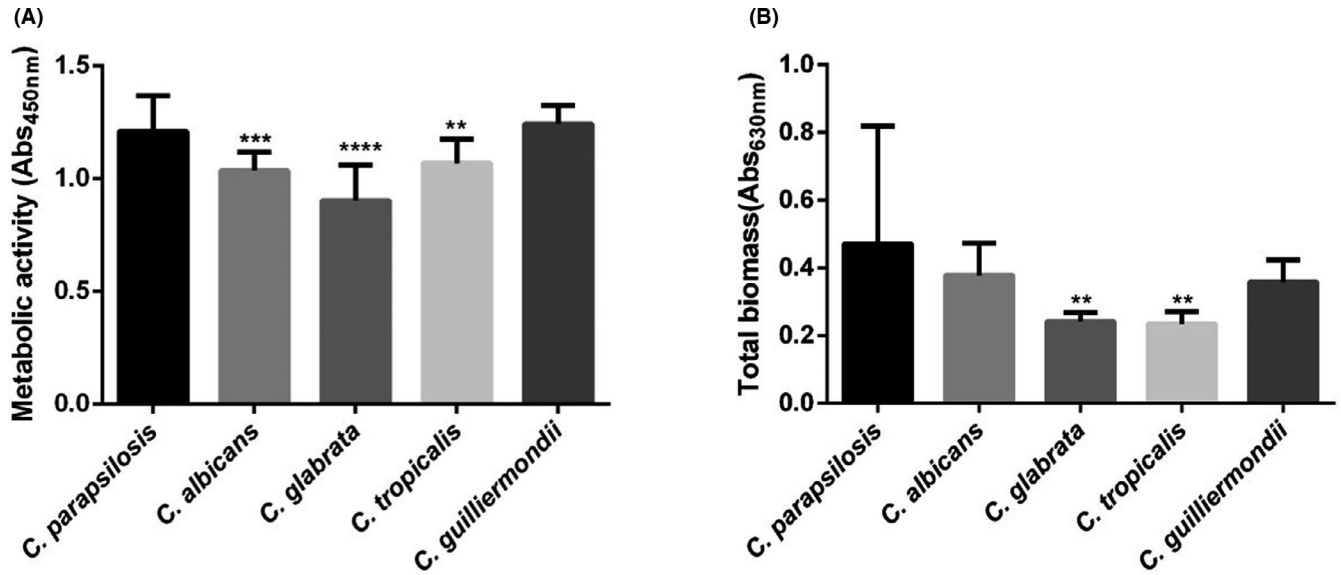
FIGURE 2 Biofilms formed by different *Candida* species at 24 h. The formation of biofilms was assessed by different colorimetric methods. (A) XTT assay, for measuring the metabolic activity of biofilms. (B) Crystal violet assay, for measuring the total biomass of biofilms. Each isolate was assessed for its biofilm-forming capability at least three times. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, versus controls
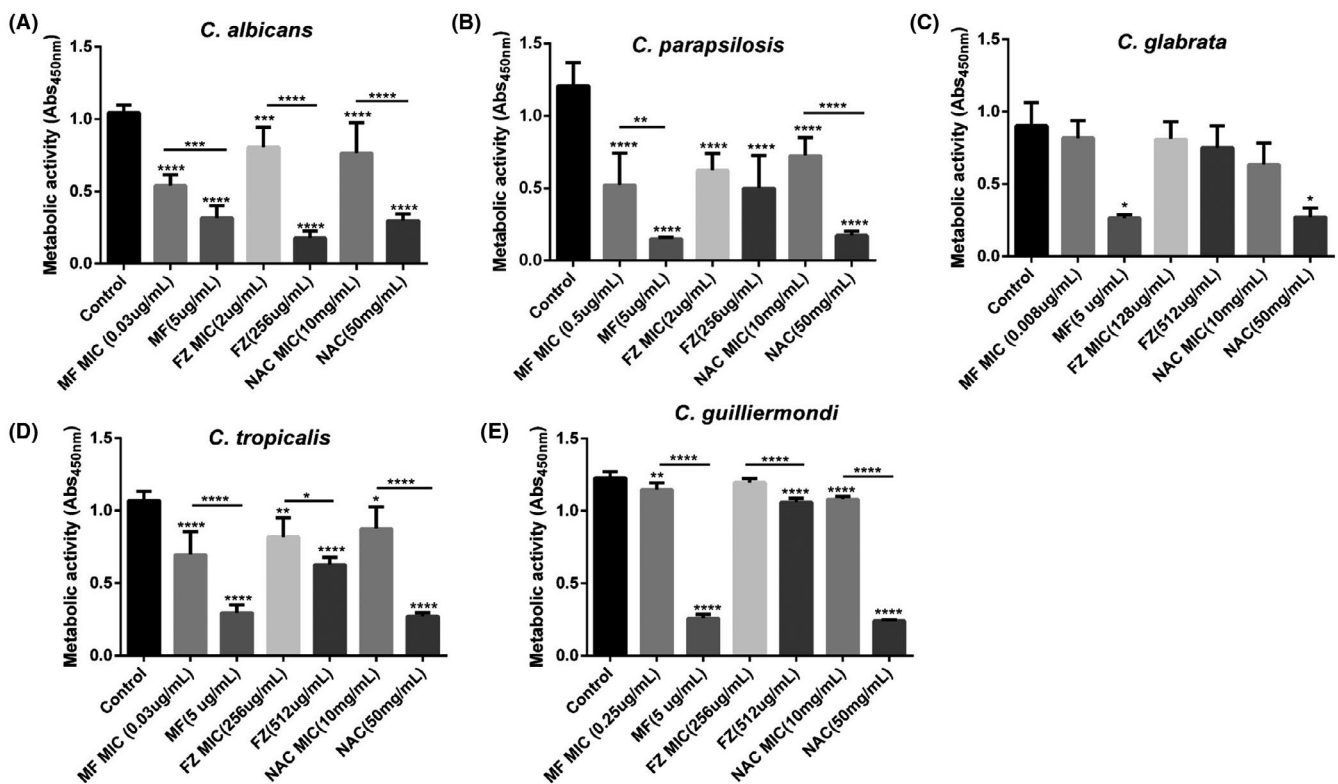

FIGURE 3 (A–E) XTT results of different *Candida* species against micafungin, fluconazole, and N-acetylcysteine at both low and high concentrations. The effects of the three antifungal agents on XRR reduction values were evaluated. Each strain was tested three times independently. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, versus controls
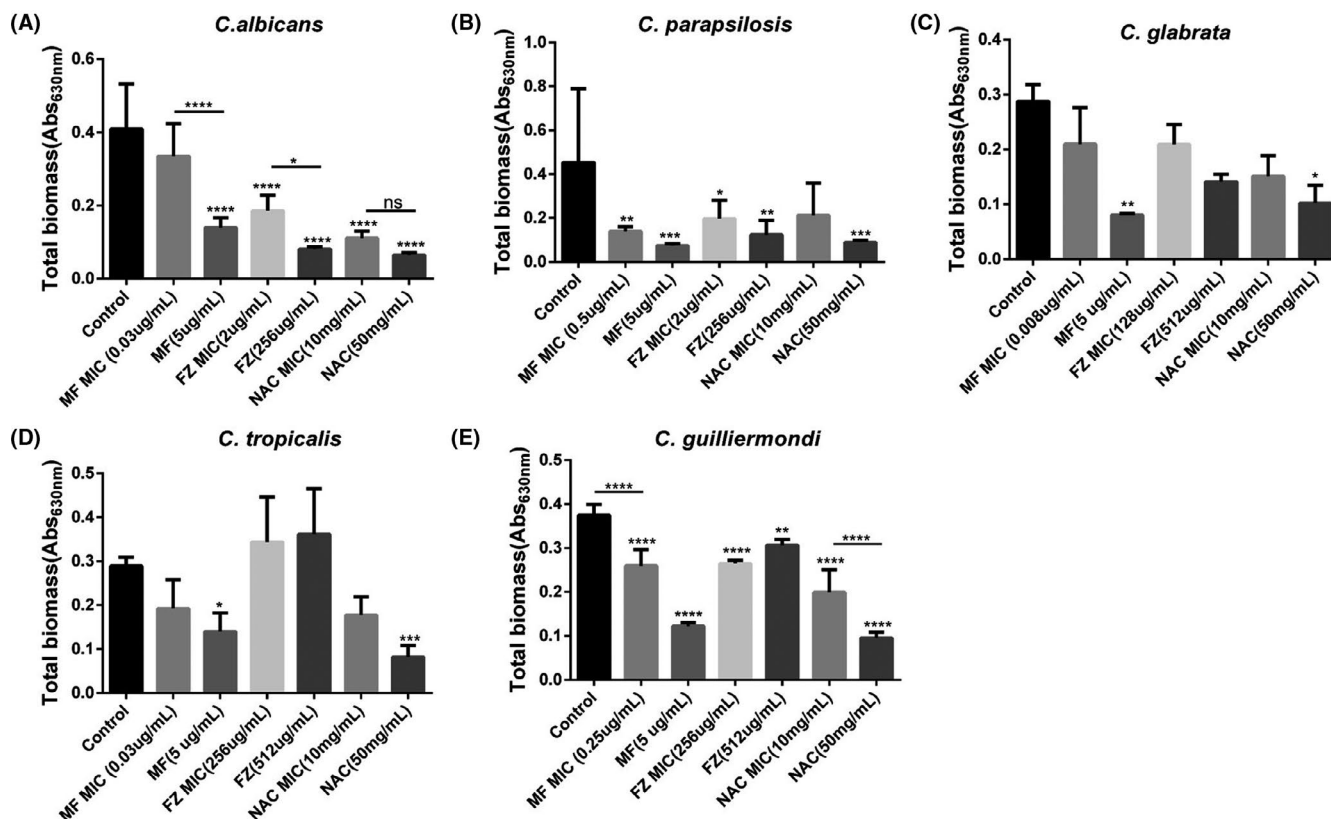
FIGURE 4 (A–E) CV results of different *Candida* species against micafungin, fluconazole, and N-acetylcysteine at both low and high concentrations. The effects of the three antifungal agents on CV values were evaluated. Each strain was tested three times independently. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, versus controls

C. tropicalis ($p < 0.01$), while there were no obvious differences in CV staining values among *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. guilliermondii*.

3.4 | XTT results of different *Candida* species against different antifungal agents

The inhibitory activities of MF, FZ, and NAC on the biofilm formation of different *Candida* species were determined by XTT assay (Figure 3A–E). For *C. albicans*, as shown in Figure 3A, when MF, FZ, and NAC were administrated at MIC concentrations, the metabolic activities of the biofilms ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$, respectively) were noticeably decreased. Interestingly, at higher concentrations, the inhibitory effects of the three antifungal agents were much stronger ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$, respectively). Similar trends could be observed for the *Candida* species of *C. parapsilosis*, *C. tropicalis*, and *C. guilliermondii*, as shown in Figure 3B,D,E, respectively. These results indicate the metabolic responses of *Candida* species to antifungal agents are concentration-dependent, especially for MF and NAC. Specifically, when the concentrations of MF and NAC were increased to 5 μg/ml and 50 mg/ml, respectively, the biofilms were disrupted nearly 4 ~ 5 folds compared to those at the lower concentrations ($p < 0.01$

and $p < 0.0001$, respectively). For the metabolic activity results of *C. glabrata*, as presented in Figure 3C, only higher concentrations of MF exhibited significant inhibitory effects compared to control group ($p < 0.05$).

3.5 | CV results of different *Candida* species against different antifungal agents

Correspondingly, the inhibitory effects of the three antifungal agents on biofilm biomass were also assessed by CV assay (Figure 4A-E). For *C. albicans* and *C. guilliermondii*, the total biomass was markedly reduced by the three antifungal agents ($p < 0.0001$). Similar to the XTT results, higher concentrations of MF induced much stronger inhibitory effects on both *Candida* species (both $p < 0.0001$), while higher concentrations of NAC only led to an obvious reduction in *C. guilliermondii* ($p < 0.0001$). For *C. parapsilosis*, all antifungal agents showed significant inhibitory effects, except for NAC at lower concentrations ($p < 0.01$, $p < 0.001$, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). However, there were no apparent differences among the tested concentrations. For *C. glabrata* and *C. tropicalis*, the marked differences were only noted after treatment with higher concentrations of MF and NAC ($p < 0.01$, $p < 0.05$ and $p < 0.001$, respectively).
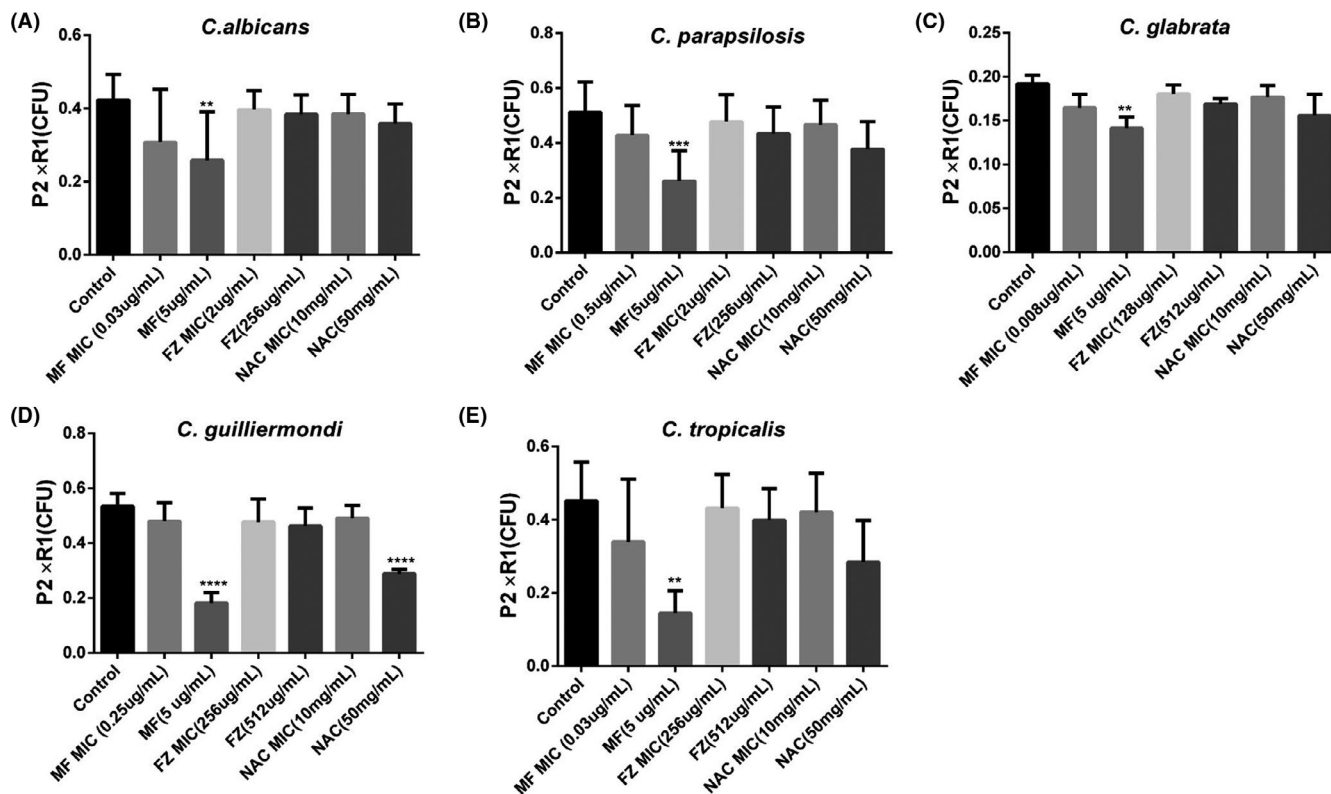
FIGURE 5 (A–E) CFU results of different *Candida* species against micafungin, fluconazole, and N-acetylcysteine at both low and high concentrations. The effects of the three antifungal agents on CFU values were evaluated. Each strain was tested three times independently. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$, versus controls

3.6 | CFU results of different *Candida* species against different antifungal agents

To determine the inhibitory abilities of the three antifungal agents on fungal adhesion, we calculated the CFUs of yeast cells that being attached to the microspheres for 24 h. The obtained value could serve as a reference standard for evaluating adhesion ability. The results indicated that the highest concentration of MF exhibited the strongest inhibitory intensity. As shown in Figure 5A–C,E 5 μg/ml of MF caused remarkable decreases in the CFUs of *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* ($p < 0.01$, $p < 0.01$, $p < 0.001$, and $p < 0.01$, respectively). As for *C. guilliermondii* (Figure 5D), the high concentrations of MF and NAC led to a marked reduction in CFUs (both $p < 0.0001$).

4 | DISCUSSION

Candida species account for the top five most common pathogens of BSIs in ICUs worldwide.[42] Although *C. albicans* is the most prominent fungal pathogen that causes candidemia in ICU patients, recent epidemiological findings have shown an increasing incidence of candidemia associated with non-*albicans* species. *C. parapsilosis* is ranked as the second relevant non-*albicans Candida* species in some areas.[43] The reason for this distribution shift has not yet been completely

understood. Based on the publicly available information, the initial adhesion is considered to be a key virulence factor for the colonization and biofilm formation of *C. albicans*. Studies about the adhesive abilities of *C. parapsilosis* have also been explored, which claimed a high intraspecies variation among clinical isolates.[35] In this study, we assessed the adhesion properties, biofilm formation, and susceptibilities to antifungal agents of 27 *Candida* species isolated from patients with CRBSIs. It was found that non-*albicans* species (e.g., *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis*) exhibited higher adhesion ability compared with *C. albicans*. Almost all *C. parapsilosis* isolates displayed a very strong profile, except for one strain (*Cpa* 5) with a strong adhesion profile (Table 2 and Figure 1). These results were in accordance with other studies [44,45] that classified non-*albicans Candida* as higher adhesion species, and *C. parapsilosis* exhibited the highest adhesion and colonization ability to biomaterials.

Another factor related to the virulence of *Candida* species is biofilm formation. We consistently characterized the attributes of all *Candida* isolates by both XTT and CV methods. XTT results indicated that *C. guilliermondii* and *C. parapsilosis* had higher metabolic activity compared to *C. albicans* (Figure 2A). CV results showed that *C. parapsilosis* generated higher amount of biomass than *C. glabrata* and *C. tropicalis* at 24 h (Figure 2B). The total biomass ranking of different *Candida* species in this study was consistent with that reported by previous studies, in which non-*albicans Candida* species were nominated as higher biomass biofilm-makers.[46] It is more important that

the results obtained from the two methods were in good agreement, except for *C. tropicals*, which had slightly different values.

After measuring the characteristics of adhesion and biofilm formation, we further explored the antifungal effects of MF, FZ, and NAC. Our findings demonstrated that the three antifungal agents caused remarkable decreases in adhesion abilities and biofilm-forming capabilities (Figures 3-5), especially MF and NAC at higher concentrations. Notably, a drastic reduction could be observed during the formation of biofilms, as revealed by both metabolic activity and total biomass.

MF, as the member of the echinocandins, is involved in the mechanism underlying the inhibition of ^{1,3}-β-D-glucan synthase. Previous research has suggested that β-1, 3 glucanase may be an important anti-biofilm candidate with a certain effect on the biofilm formation process of non-*albicans Candida* species.[47] Cateau et al[38] demonstrated that MF (5 mg/L) had the ability to inhibit the metabolic activity of *C. albicans* during biofilm growth. Moreover, some studies also found that MF exhibited inhibitory activity on the biofilm formation of *C. parapsilosis*.[48] In our study, MF also played essential roles in the adhesion and biofilm formation of non-*albicans Candida* species. Nevertheless, the mechanism of action is still unclear, which requires further investigation.

Although NAC is commonly known as a non-antibiotic drug, its antibacterial properties have also been reported.[49-51] Interestingly, many studies shown that NAC was able to suppress both adherence and mature biofilms formed by *C. albicans*, which makes NAC an interesting candidate for inhibiting biofilm formation.[40,41,52] However, the effect of NAC on non-*albicans Candida* species is still relatively unknown. In this study, the inhibitory effects of NAC on clinical non-*albicans Candida* isolates were investigated, and the results showed that NAC could reduce both adhesion and biofilm formation. More specifically, its anti-biofilm activity seems to be concentration-dependent. Besides, a previous report [53] indicated that NAC acted synergistically with other antimicrobial agents such as tigecycline. Consistently, our findings also showed that NAC had antifungal properties. Regarding the mode of action, some authors proposed that the reduction of adhesion by NAC was chemical as well as biological,[52] while others postulated that NAC could inhibit the biofilm growth and affect the texture of biofilms formed by *C. albicans*.[41,51] These encouraging findings still need to be verified on non-*albicans Candida* species through both in vitro and clinical studies in the near future.

Several limitations are existed in our study. First, due to the low incidence of CRBSIs, the sample size of clinical isolates in this study was relatively small. For more convincing results, we intended to expand the study by collecting more clinical isolates and examining the correlation between adhesion abilities and biofilm-forming capabilities. Second, there was a lack of morphological data in the present experiment. Fluorescence inverted microscope or laser confocal microscope can be used to detect the morphological changes in biofilm formation with or without antifungal treatment. Third, our study only proved the inhibitory effect of a single antifungal agent, without the combination of other antifungal agent. Interestingly, some of our unpublished data showed that the combination of NAC and MF exhibited stronger inhibitory effects on the biofilm formation of *Candida* species. Finally, the molecular and genetic mechanisms need to be elucidated in the future studies. For example, a phylogenetic tree analysis can be performed on these *Candida* species in order to find some clues by linking the distance of these organisms with the results of biofilm formation.

5 | CONCLUSION

In this study, we observed that non-*albicans Candida* species (e.g., *C. guilliermondii*, *C. tropicalis*, and *C. parapsilosis*) demonstrated higher adhesion abilities, while their biofilm-forming capabilities varied across species. For antifungal therapy, MF was shown to have stronger inhibitory effects on the adhesion and biofilm formation of different *Candida* species in a dose-dependent fashion. Our study also verified that NAC had antifungal potency for reducing both adhesion and biofilm formation. These findings can help clinicians better understand the pathogenesis of catheter-related candidemia and treat such type of infections.

CONFLICT OF INTEREST
The authors declare that they have no conflicts.

AUTHOR CONTRIBUTIONS
KH and LYZ conceived the project and designed the study, XC and YNL analyzed and interpreted the data. XSZ drafted the primary draft of the manuscript. All authors revised and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
All data are provided in full in the Results section of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.