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Comparative virulence traits of Candida spp. in pregnant and non-pregnant women with vulvovaginal candidiasis

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Abstract

Vulvovaginal candidiasis (VVC) is highly prevalent in women of reproductive age and represents an important public health concern. Its high incidence and recurrence, together with antifungal resistance and therapeutic limitations during pregnancy, underscore the need for a better understanding of *Candida*-related phenotypes. By identifying the predominant *Candida* species causing VVC, this study aimed compare antifungal susceptibility and virulence-associated phenotypes among isolates from pregnant and non-pregnant women. *Candida* spp. isolates were obtained from women with VVC who were pregnant (G1), non-pregnant (G2), or non-pregnant with recurrent VVC (G3). Species identification was performed using MALDI-TOF mass spectrometry. Antifungal susceptibility was evaluated using standardized testing, while biofilm formation and metabolic activity were assessed by *in vitro* assays. *In vivo* virulence was evaluated using the *Tenebrio molitor* model. A total of 29 isolates were recovered, predominantly *Candida albicans*, followed by *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis*. VVC was more frequent in pregnant women than non-pregnant women. Although antifungal susceptibility varied between isolates, some exhibited resistance or reduced susceptibility to one or more drugs, particularly azoles. Biofilm phenotypes also varied, with alterations in biomass production and metabolic activity observed across isolates. G1 isolates induced high mortality rates in the *T. molitor* model, indicating pronounced *in vivo* virulence. Overall, these findings highlight the predominance of *C. albicans* in VVC, including pregnancy women and recurrent cases, and demonstrate heterogeneous antifungal susceptibility alongside by distinct biofilm and virulence phenotypes. The marked virulence observed in G1 and biofilm alterations in G1, G2, and G3 underscore the complex pathogenic profiles of VVC isolates.

Introduction

Fungal infections are a challenge to global public health [1, 2]. Involving climate change, the increasing prevalence of immunosuppression in the population, along with the emergence of antifungal resistance mechanisms, contributes to a particularly complex landscape in the management of these diseases [3–5].

Candida species stand out in the context of fungal infections owing to their significant role in the etiology of vulvovaginal candidiasis (VVC), which is a highly prevalent mycosis among women worldwide [6]. It is estimated that 75% of women will experience at least one episode in their lifetime, with the highest incidence occurring during the reproductive years [7]. During pregnancy, the prevalenceis pronounced, increasing by approximately 20% to 30% due to the hormonal and immunological changes characteristic of this physiological state [8, 9].

Candida albicans remains the species most associated with VVC [10, 11]. Nevertheless, other clinically relevant species, including *Candida glabrata*, as well as less frequently isolated species, such as *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei*, may also be responsible for the disease [11–13]. Multifactorial factors contribute to the colonization and infection of these species involved in VVC,

including genetic predisposition, uncontrolled diabetes, inappropriate use of antibiotics, and immunosuppression [14–16].

Treating VVC is difficult, time-consuming, and economically burdensome [17]. Current treatment options are insufficient, with a limited number of antifungal targets and drug-related constraints [17, 18]. The antifungal therapy depends on a range of factors, including the *Candida* species involved, the anatomical site of infection, disease severity, and the immune status of the patient [19].

In addition, the main limitation to clinical success lies in the progressive emergence of resistant phenotypes, inherent resistance to certain antifungals, and the presence of recurrent infections [20]. Recurrent VVC (RVVC) introduces an additional degree of complexity in disease management [21]. In this clinical condition, affected woman experience one or more episodes within a one-year period, representing a significant clinical hurdle in treatment [22]. RVVC is estimated to affect approximately 70% of women who experience VVC at least once in their lifetime [21–23].

Furthermore, fungal biofilm formation represents a multifactorial barrier to successful antifungal therapy [24]. Biofilms impair antifungal penetration, promote structural modifications or the overexpression of drug targets, and activate efflux mechanisms mediated by membrane transporters [25].

Given this context, investigating the mechanisms involved in antifungal resistance in VVC is crucial for understanding the dynamics between the host and pathogen, which may lead to the development of more effective and targeted therapeutic strategies [26]. In this context, the pursuit of alternative experimental models is essential for advancing research on VVC. Simplified *in vivo* systems, such as *Tenebrio molitor* larvae, provide a valuable alternative for assessing fungal virulence and host–pathogen interactions, offering greater experimental simplicity, lower costs, and reduced ethical concerns while minimizing the use of conventional animal models [27].

Accordingly, the present study aimed to identify the main *Candida* species responsible for VVC in clinical samples and to characterize their antifungal resistance and virulence profiles in a comparative analysis between pregnant and non-pregnant women *in vitro* and *in vivo*.

Methods

Sample acquisition

Clinical samples were collected by cervicovaginal swabs (Onlen, Yangzhou, Jiangsu Province, China) from 107 women (25 pregnant and 82 non-pregnant) attending Hospital das Clínicas of the Ribeirão Preto Medical School, University of São Paulo (HCRP-USP), Brazil, between March and September 2025. Swabs were inoculated onto Sabouraud dextrose agar (SDA) supplemented with chloramphenicol (0.5 g/L; Kasvi, Pinhais, PR, Brazil) and incubated at 35°C for 48 h. Species-level identification was performed at HCRP-USP using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) were determined in accordance with CLSI guidelines [28]. Antifungals were tested at 0.01–64 µg/mL for amphotericin (AMB), itraconazole (ITC), ketoconazole (KTZ), miconazole (MCZ), and voriconazole (VRC), and 0.01–256 µg/mL for fluconazole (FLC) and terbinafine (TRB). Plates were incubated at 35°C and read visually at 48 h. MIC was defined as complete inhibition (MIC100) for AMB and TRB or 50% growth reduction (MIC50) for the other agents. *C. albicans* ATCC 64548 and *C. parapsilosis* ATCC 22019 as control strains.

/ n vitro biofilm biomass and metabolic activity

The biofilms were prepared as previously described [27], with modifications. Biofilm biomass was quantified by optical density (OD) after crystal violet staining (100 µL). Biofilm production was classified based on the OD cutoff (ODc), calculated as the negative control mean OD plus three standard deviations ($ODc=OD_{nc} + 3 \times SD_{nc}$) as previously described [29]. The ODc for this experimental set was 0.0626 (Table S1). Isolates were classified as non-producers ($OD_{550} \leq 0.0626$), low producers ($0.0626 < OD_{550} \leq 0.1252$; ≤2×ODc), intermediate producers ($0.1252 < OD_{550} \leq 0.2504$; ≤4×ODc), or high producers ($OD_{550} > 0.2504$; >4×ODc). The OD_{nc} and SD_{nc} values used to calculate the ODc are provided in Table S1[30].

For MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, after incubation, wells were washed again with PBS, and 2 µL of menadione and 20 µL of MTT (Sigma-Aldrich; final concentration, 5000 µg/mL) were added to each well [31]. Results were expressed as OD values, with experiments performed in biological triplicate. Metabolic activity was classified as low (LMA), intermediate (IMA), or high (HMA) based on the first (T1) and second (T2) tertiles of the MTT absorbance values, as previously described [32]. T1 and T2 were defined as the observed values equal to or closest to the tertile cutoffs of 33.3% and 66.7%, respectively.

Survival assay in an alternative model

The survival assay was performed following [27], with modifications. Larvae weighing 150–200 mg, with uniform coloration and no dark spots, were selected and injected using a Hamilton syringe (701 N, 26 gauge). Groups of 10 larvae received either PBS (50 mL/kg; control) or the respective inoculum (50 mL/kg). Isolates showing pronounced biomass and metabolic activity, alongside elevated MIC values were selected, along with strains *C. albicans* ATCC 64548 and *C. parapsilosis* ATCC 22019. Larvae were incubated at 35°C with a rearing diet, and mortality was recorded daily for 20 days. Survival was expressed as the percentage of live larvae per day and analyzed using Kaplan–Meier curves.

Statistical analysis

Results are expressed as means ± standard deviations of three independent biological replicates. Biofilm metabolic activity and biomass were compared using one-way ANOVA with Tukey's post hoc test or the

Kruskal–Wallis test. Survival curves were analyzed using the log-rank test with Bonferroni-adjusted pairwise comparisons. Statistical significance was set at $p < 0.05$. Analyses and graphs were generated using GraphPad Prism v. 11.1 (GraphPad Software, San Diego, CA, USA).

Results

Prevalence and species distribution of Candida spp. in pregnant and non-pregnant women

A total of 29 yeast isolates with phenotypic characteristics consistent with those of *Candida* spp. were recovered, including 23 (79.3%) *C. albicans* (*Ca*0007, *Ca*0009, *Ca*0012, *Ca*0014, *Ca*0021, *Ca*0022, *Ca*0025, *Ca*0038, *Ca*0050, *Ca*0051, *Ca*0059, *Ca*0060, *Ca*0061, *Ca*0062, *Ca*0065, *Ca*0066, *Ca*0078, *Ca*0079, *Ca*0099, *Ca*0100, *Ca*0105, *Ca*0108, and *Ca*0109), and six non-*albicans Candida* species were identified, comprising three *C. glabrata* strains (*Cg*0041, *Cg*0042, and *Cg*0063), two *C. parapsilosis* strains (*Cp*0092 and *Cp*0098), and one *C. tropicalis* strain (*Ct*0074). No *C. krusei* isolates were detected in the studied cohort.

Among pregnant women, 32% (n = 8/25) were positive for infection by at least one *Candida* species, whereas a lower frequency was observed among non-pregnant women, in whom 25.6% (n = 21/82) tested positive. A 6.4% increase in the frequency of *Candida* spp. infection among pregnant women compared with non-pregnant women (Fig. 1a).

When stratified by pregnancy status, seven pregnant participants (n = 25) were positive for *C. albicans* (28%), and one was positive for *C. glabrata* (4%), with no other species detected. In the cohort of non-pregnant women (n = 82), 16 patients were positive for *C. albicans* (19.5%), two for *C. parapsilosis* (2.4%), two for *C. glabrata* (2.4%), and one for *C. tropicalis* (1.2%) (Fig. 1b).

Antifungal susceptibility of Candida spp.

Four groups were established for antifungal susceptibility and biofilm analyses: pregnant patients (G1; n = 8), non-pregnant patients with VVC (G2; n = 13) or RVVC (G3; n = 8), and reference strains *C. albicans* ATCC 64548 and *C. parapsilosis* ATCC 22019 (RS). In G1, most isolates showed low MICs for AMB, ITC, KTZ, and MCZ, with *Ca*0038 and *Ca*0060 presenting values ≤ 0.06 µg/mL. In contrast, *Ca*0007, *Ca*0012, *Ca*0014, *Ca*0038, and *Cg*0041 showed FLC MICs > 0.5 µg/mL, while all *C. albicans* isolates had VRC MICs > 0.03 µg/mL (0.06–2 µg/mL). *Ca*0060 showed the lowest TRB MIC (4 µg/mL), whereas *Ca*0007 had the highest ITC MIC (16 µg/mL).

In G2, several isolates showed elevated MICs across multiple agents. *Ca*0022 and *Ca*0065 exhibited reduced susceptibility to all antifungals tested, with *Ca*0065 showing an FLC MIC of 16 µg/mL; *Ca*0022 had MICs of 8 µg/mL for KTZ, AMB, and FLC. *Ca*0025 showed an AMB MIC > 8 µg/mL, while *Ca*0061 and *Cg*0063 presented the highest TRB MICs (128 µg/mL).

In G3, the highest MICs were mainly observed for TRB, reaching 256 µg/mL in *Ca*0100 and *Ca*0108 and 128 µg/mL in *Ca*0105. *Ca*0105, *Ca*0108, and *Ca*0109 also showed elevated MICs for multiple azoles,

particularly FLC (8, 16, and 16 µg/mL, respectively). Overall, AMB MICs were lower in G3 (0.12–1 µg/mL). MIC50 and MIC100 values are presented in Table S2.

Biofilm biomass quantification of Candida spp.

All the experimental groups exhibited a high capacity for biomass formation, with OD_{550} values exceeding 0.2504. Within G1, most isolates exhibited biomass levels comparable to those of the reference strains. In this context, isolates *Ca*0007, *Ca*0012, *Ca*0050, *Ca*0051, and *Ca*0060 are noteworthy, as they presented optical density (OD_{550}) values close to 3. Despite these variations in absolute OD values, statistical analysis revealed no significant differences among the isolates within G1 (Fig. 2a).

In G2, *Ca*0066, *Ct*0074, *Ca*0078, *Cp*0092, and *Cp*0078 showed biofilm biomass above the reference threshold, with OD_{550} values approaching 4. The remaining isolates also exhibited high biomass, but at significantly lower levels than these isolates. *Ca*0025 showed the lowest biomass in G2, although its OD_{550} remained within the high biomass-producing range (Fig. 2b).

The isolates belonging to G3 presented markedly high biofilm biomass production. Among the eight evaluated isolates, five (*Ca*0059, *Ca*0079, *Ca*0099, *Ca*0100, and *Ca*0105) presented optical density values exceeding the threshold established based in the RS, with readings close to 4. Within this group, the isolate *Ca*0009 displayed a relatively lower biomass level, with an OD value of approximately 3. Nevertheless, this result remained close to the reference values used as a comparative benchmark and higher than 0.2504. Similarly, *Ca*0108 and *Ca*0109 produced biomass below the reference values and below those of the other isolates. Nevertheless, they were still classified as strong biomass producers (Fig. 2c).

G1 showed a broad, asymmetric distribution, with most values between ~ 2.2 and 2.8 and one pronounced value, whereas G2 was the most homogeneous, with tightly clustered values and one high outlier. Both groups had identical means, aligned with the lower reference value (2.54). G3 had the highest mean, close to the upper reference value (3.36), but showed the greatest heterogeneity (Fig. 2d).

Metabolic activity assessment of Candida spp.

Biofilm metabolic activity was stratified based on cutoff values defined by the first (T1) and second (T2) tertiles of the absorbance distribution, as calculated in Table S3. The cutoff values for three groups are shown in Table S4.

Among the G1 isolates, only three presented HMAs: *Ca*0007, *Ca*0050, and *Ca*0051. These isolates presented comparable metabolic levels, with no statistically significant differences between them, and both exceeded the activity observed in the RS. Two isolates exhibited IMA (*Ca*0012 and *Ca*0038), whereas three showed LMA (*Ca*0014, *Cg*0041 and *Ca*0060) (Fig. 3a).

G2 isolates showed an IMA profile. *Cg*0042, *Cg*0063, and *Ca*0065 exhibited the highest metabolic activity, with values approximately twofold above the reference thresholds, while *Ca*0066 also showed an

HMA profile, but at a lower magnitude. No significant differences were observed among the three most metabolically active isolates. In contrast, *Ca*0021, *Ca*0025, *Ca*0061, *Ca*0062, *Ca*0078, and *Cp*0092 showed IMA, with values close to the reference thresholds, whereas *Ca*0022, *Ct*0074, and *Cp*0098 exhibited LMA. Notably, *Ct*0074 showed one of the lowest metabolic activity levels among the isolates (Fig. 3b).

G3 isolates showed significant variation in biofilm metabolic activity. *Ca*0059, *Ca*0108, and *Ca*0109 were classified as HMA, with *Ca*0108 and *Ca*0109 showing higher values than the other isolates. In contrast, *Ca*0079, *Ca*0100, and *Ca*0105 showed IMA, whereas *Ca*0009 and *Ca*0099 exhibited LMA (Fig. 3c).

Metabolic activity overlapped substantially across groups, with greater dispersion in G1 and G2, particularly the latter, where several isolates exceeded the upper reference threshold. G3 showed a narrower distribution, with most values within the reference range. Despite inter-isolate variability, central values remained near the reference thresholds, with no significant differences among groups (Fig. 3d).

In vivo evaluation of Candida spp. virulence

Within 11 days post-infection, all G1 isolates caused 90% mortality in *T. molitor* larvae. *Ca*0012 reached this endpoint within 7 days, whereas *Ca*0051 and *Ca*0007 required 10 and 11 days, respectively. The RS ATCC 64548 and ATCC 22019 caused 70% and 80% mortality, respectively. Despite these differences in mortality rates, no statistically significant differences in larval survival were observed between the RS and the three G1 isolates (Fig. 4a).

G2 isolates showed lower virulence than the reference strains, with mortality rates of 40% for *Cg*0042 and *Ca*0065 and 30% for *Cg*0063. The latter was significantly less virulent than both RS, whereas *Cg*0042 and *Ca*0065 differed significantly only from ATCC 22019 (Fig. 4b). Among G3 isolates, *Ca*0108 mirrored the survival pattern of *Ca*0012, reaching 90% mortality by day 9 post-infection. *Ca*0109 caused 70% mortality, comparable to ATCC 64548, whereas *Ca*0105 caused 50% mortality, exhibiting the highest larval survival and lowest virulence in this group. No significant differences in larval survival were detected among G3 isolates (Fig. 4c). G1 exhibited the lowest larval survival and highest apparent virulence, with no significant difference from G3. Conversely, G2 showed the highest survival (~ 40%), indicating the lowest apparent virulence. Pairwise comparisons revealed significant survival differences between G1 and G2 and between G2 and G3 (Fig. 4d).

Discussion

Our study confirmed the predominance of *C. albicans* across all groups and a higher frequency of VVC among pregnant women, despite their lower representation in the group. *C. albicans* was the sole species recovered in RVVC, underscoring its role in acute and recurrent disease, whereas non-*albicans* *Candida* species were less frequent [33, 34]. Antifungal susceptibility profiling is essential to characterize the therapeutic response of clinically relevant isolates amid etiological and phenotypic heterogeneity [35].

The G1 isolates showed heterogeneous azole susceptibility, with clinically relevant resistance to FLC in *Ca*0007, and *Ca*0014 and to VRC in *Ca*0012 and *Ca*0014, according to current CLSI breakpoints. *Ca*0051 was intermediate to VRC. The concurrent resistance to multiple azoles observed in *Ca*0014 may reflect the involvement of shared mechanisms underlying azole resistance and, consequently, may reduce the therapeutic options available for infections caused by this isolate [36]. *Ca*0007 and *Ca*0014 exhibited higher MICs to ITC and MCZ, respectively. Although these values did not fulfil CLSI resistance criteria, they suggest isolate-specific susceptibility shifts that warrant further investigation.

The resistance observed among isolates from pregnant women may reflect the interplay between pregnancy-associated hormonal and immunological changes and repeated exposure to azole antifungals, particularly FLC [37, 38]. These conditions may promote persistent *Candida* spp. colonization and repeated antifungal exposure, exerting selective pressure that favors the emergence and persistence of less susceptible phenotypes through established resistance mechanisms [8].

In G2, *Ca*0022 and *Ca*0065 were resistant to FLC and VRC. The variability in susceptibility to the other antifungal agents, particularly TRB, may indicate differences in the mechanisms underlying antifungal response among G2 isolates. The predominance of clinically defined resistance among azoles highlights the relevance of this antifungal class in the susceptibility profile of G2 isolates. In G3, the azole susceptibility profile was even more heterogeneous, with a higher concentration of clinically resistant phenotypes compared with G1 and G2. Five isolates exhibited resistance to VRC (*Ca*0009, *Ca*0100, *Ca*0105, *Ca*0108, and *Ca*0109), while three were resistant to FLC (*Ca*0105, *Ca*0108, and *Ca*0109).

Taken together, a consistent and clinically relevant shift toward reduced susceptibility across the azole class. This pattern is consistent with reports of azole cross-resistance, largely attributable to shared molecular targets and overlapping resistance mechanisms [39–41]. The resistance to FLC has increased over time, particularly among isolates recovered from recurrent or persistent infections, a trend commonly linked to prolonged or repeated exposure to azole therapy [42–44].

This finding is particularly relevant in the context of the pronounced biofilm-forming capacity observed among the isolates, as biofilm growth can further reduce antifungal susceptibility and compromise therapeutic efficacy [45–47].

The isolates exhibited substantial biofilm-forming capacity, with biomass values exceeding the established cutoff (OD_{550} > 0.248). This phenotype was widespread and may promote infection persistence and reduced antifungal susceptibility. Except for *Ca*0009, *Ca*0014, and *Ca*0022, all isolates resistant to at least one antifungal displayed high biomass and intermediate-to-high metabolic activity.

G3 showed the greatest biomass production, with most isolates classified as strong biofilm producers and values approaching or exceeding those of the reference strains. Most also exhibited moderate-to-high metabolic activity, which, combined with high biomass, may contribute to the elevated MICs observed.

The coexistence of dense and metabolically active biofilms is known to confer increased tolerance to antifungal agents, providing a plausible explanation for the reduced susceptibility profiles observed [48, 49]. In the case of G3, these characteristics may be directly linked to the enhanced ability of these isolates to establish and sustain RVVC, further underscoring the central role of biofilm formation as a key determinant of virulence and therapeutic failure.

The *in vivo* infection revealed a highly virulent phenotype among the G1 isolates. In absolute terms, mortality observed in this group was higher than that recorded for G3, although the latter was initially expected to exhibit greater virulence. Particularly in G1, these isolates exhibited high biomass and intermediate-to-high metabolic activity, potentially favoring biofilm persistence and adaptation. In both groups, most isolates with the highest mortality rates also showed elevated MICs to multiple antifungals, with only one exception.

In conclusion, VVC was predominantly associated with *C. albicans*, including recurrent cases and infections during pregnancy. The isolates exhibited reduced antifungal susceptibility, particularly to azoles, with FLC showing the most concerning profile, potentially reflecting cumulative therapeutic exposure; less frequent alterations in susceptibility to non-azole agents suggest broader adaptive mechanisms. All groups exhibited concerning virulence profiles, with G1 showing the highest *in vivo* virulence, underscoring the pathogenic potential of pregnancy-associated isolates.

Declarations

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Ethics approval: This study was conducted in accordance with approval from the Ethics Committee of the University Hospital of Ribeirao Preto Medical School, USP, Brazil (HCFMRP-USP), under Protocol No. 8.183.835.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

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Figures

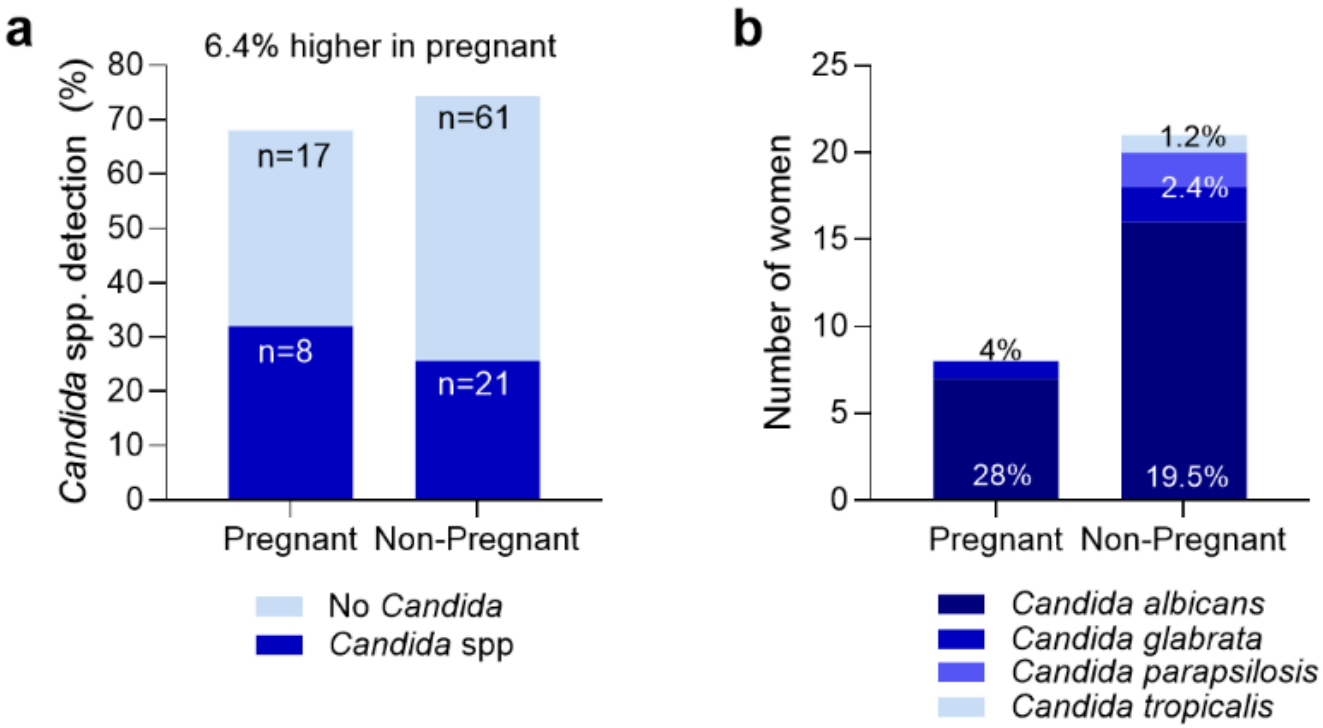


Figure 1

Prevalence and species distribution of *Candida* in pregnant and non-pregnant women. (**a**) Percentage of pregnant and non-pregnant women with and without *Candida*spp. detection. (**b**) Distribution of *Candida* species identified among women with *Candida* spp. detection in each group. Values within the bars indicate the number of women (n)

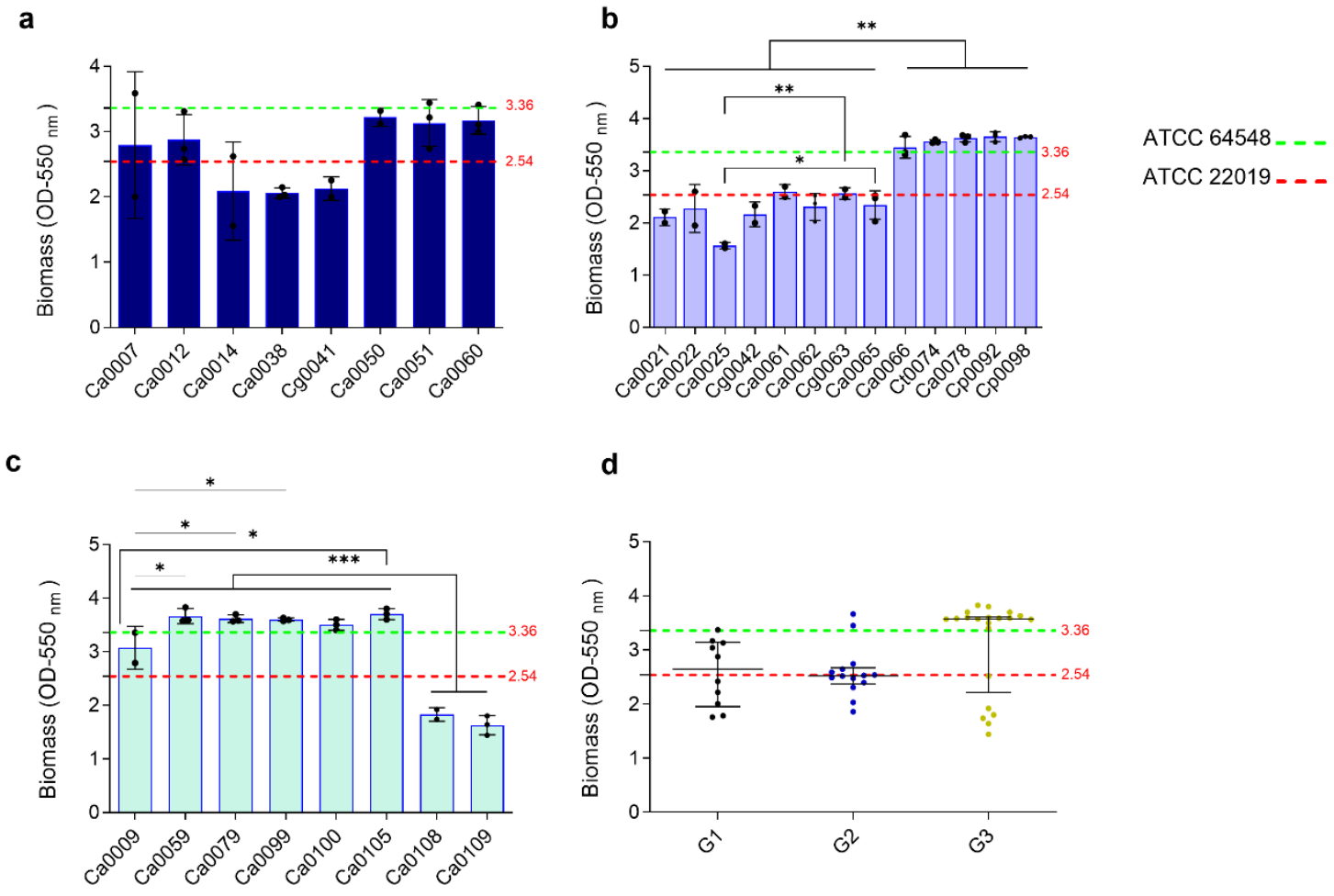


Figure 2

Quantitative assessment of *in vitro* biofilm biomass production by *Candida* spp. isolates. (**a**) Biofilm biomass production by G1, (**b**) G2, and (**c**) G3 isolates. (**d**) Comparison of biofilm biomass production among the three clinical groups. The green and red dashed lines indicate thresholds derived from the mean absorbance values of ATCC 64548 and ATCC 22019, respectively. Statistical significance was assessed via one-way ANOVA followed by Tukey's post hoc test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

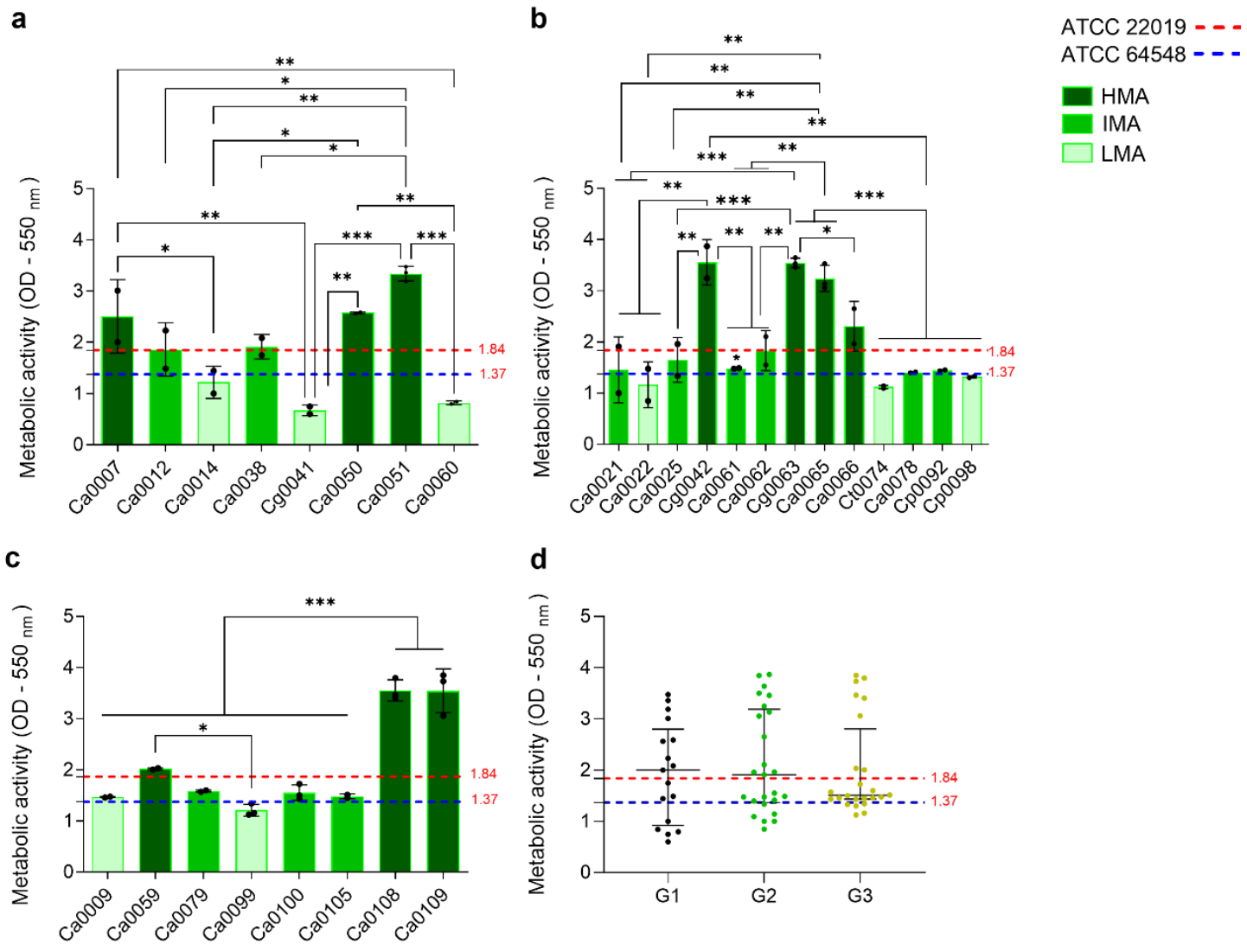


Figure 3

Group-based differences in the metabolic activity of biofilms of clinical *Candida* spp. isolates. (**a**) Biofilm metabolic activity of G1, (**b**) G2, and (**c**) G3 isolates. (**d**) Comparison of biofilm metabolic activity among the three clinical groups. The green and red dashed lines indicate thresholds derived from the mean absorbance values of ATCC 64548 and ATCC 22019, respectively. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$; *$p < 0.01$; **$p < 0.001$). The asterisk above *Ca*0061 indicates a significant difference from *Cg*0063

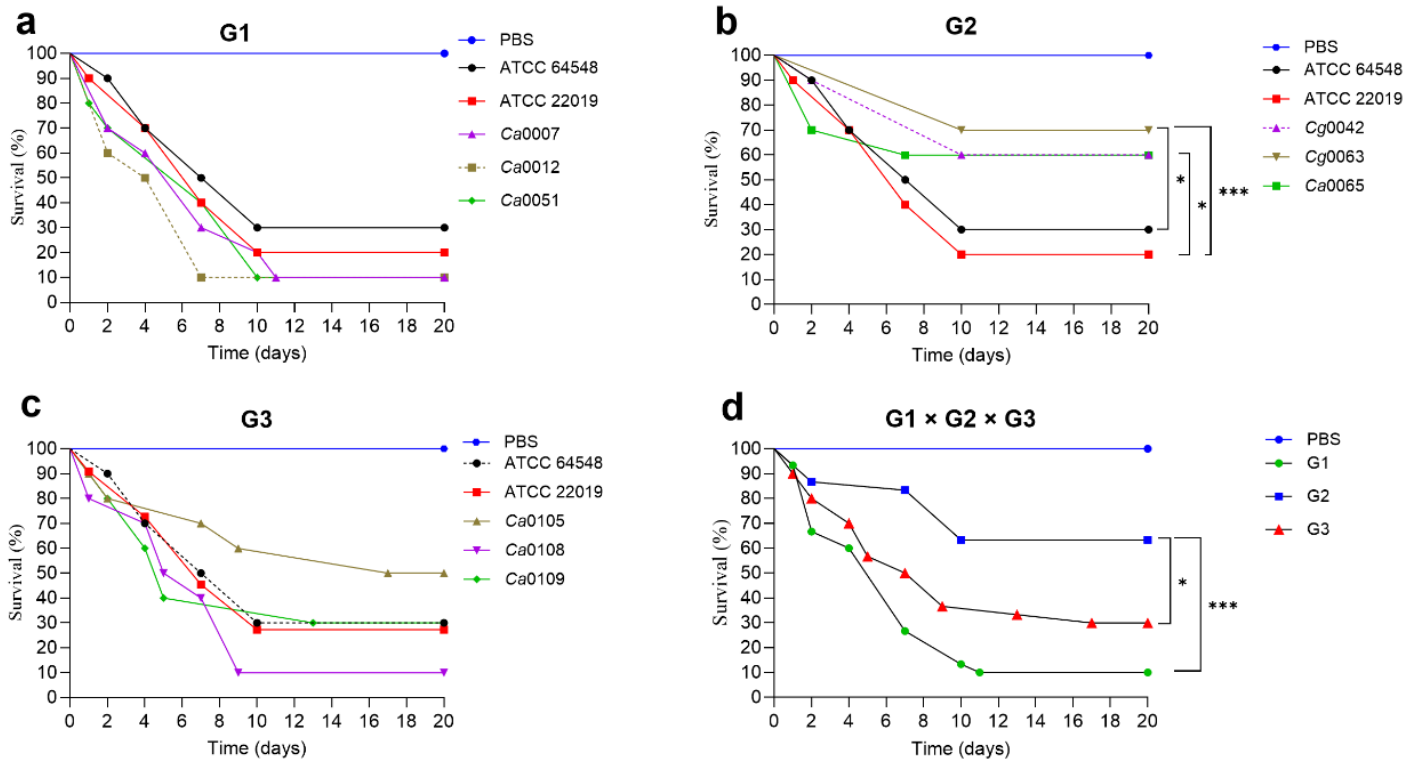


Figure 4

In vivo virulence assessment of *Candida* spp. isolates using *Tenebrio molitor* larvae. (**a**) Survival of larvae infected with G1, (**b**) G2, and (**c**) G3 isolates. (**d**) Comparison of larval survival across the three experimental groups. Statistical analysis was performed using the log-rank (Mantel–Cox) test, with pairwise comparisons adjusted using the Bonferroni test (* $p < 0.05$; *** $p< 0.001$)

Supplementary Files

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