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Antibiofilm and antiproliferative effect on *Candida* spp. generated by the combination of conventional antifungals

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Abstract

Invasive infections caused by *Candida* spp. and biofilm-associated resistance necessitate novel therapeutic strategies. Currently, combining conventional antifungal agents with distinct mechanisms of action represents a promising approach. This study evaluated the antibiofilm and antiproliferative potential of caspofungin (CAS), isavuconazole (ISA), itraconazole (ITR), amphotericin B (AMB), and ciprofloxacin (CIP), alone and in combination, against 24 h mature biofilms of four *Candida* species. Initially, the morphological characteristics and molecular identity of four *Candida* spp. isolates were confirmed, and their biofilm-forming capacity was determined. Subsequently, microdilution assays were performed to determine the Fractional Inhibitory Concentration Index (FICI), and the XTT reduction assays were used to assess metabolic activity. Additionally, confocal laser scanning microscopy was used to analyze biofilm architecture and thickness in each treatment. Morphological characteristics and molecular data confirmed the identity of *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (*Pichia kudriavzevii*), as well as their ability to form biofilms. After evaluating possible antifungal combinations, the CAS + ISA (25 + 2 µM) and CAS + AMB (25 + 10 µM) combinations demonstrated a greater capacity to alter biofilm architecture than either treatment alone. Thus, metabolic, and microscopic assays revealed a significant antibiofilm and inhibitory effect in the *Candida* species studied. The combination of echinocandins with azoles or polyenes exerts a critical disruptive effect on the *Candida* spp. biofilm, overcoming the resistance observed with individual treatments. The combinations CAS + ISA and CAS + AMB at optimized concentrations are emerging as strategies, particularly effective against biofilms of non-albicans species

such as *C. tropicalis*.

Keywords: Candidiasis¹, Synergism², Exopolysaccharides³, Echinocandins⁴

Introduction

Invasive mycoses caused by *Candida* species, commonly known as Candidiasis, represent a critical challenge for global public health, with mortality rates exceeding 60% in patients with systemic infections despite the availability of antifungal treatment (Mantilla-Florez et al. 2021). In this context, *C. albicans* remains the main etiological agent; however, the epidemiology has shifted toward non-*albicans* such as *C. tropicalis*, *C. parapsilosis* and *C. krusei* (Malinová et al. 2023), which have been classified by the World Health Organization (WHO) as high- and medium-priority fungal pathogens (World Health Organization 2022). Biofilm formation in *Candida* spp., is a determinant virulence factor in the pathogenesis and persistence of infection (Arafa et al. 2023). Biofilms are three-dimensional communities embedded in a matrix of extracellular polymeric substances (EPS) (Walter et al. 2025), conferring intrinsic resistance to the host immune response and conventional antifungals upon the fungal cells (Ajetunmbi et al. 2023). Furthermore, intrinsic and acquired resistance within biofilms involves mechanisms such as overexpression of efflux pumps, alterations in membrane permeability, and the presence of persisted cells with reduced metabolism, all of which represent a major challenge for conventional antifungal therapy (Nadar et al. 2022).

In clinical practice, treatment for the different forms of candidiasis is based primarily on the use of polyenes, azoles, and echinocandins (Mantilla-Florez et al. 2021), specifically, amphotericin B (AMB) is a polyene that has been used for more than 50 years and is considered a first-line antifungal option for *C. albicans*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* (Martínez-Méndez et al. 2023). Among the echinocandins, caspofungin (CAS) has been commercially available since 2001 and was originally designed to treat Candidiasis, it is currently a first-line option for neutropenic patients (Hoenigl et al. 2021), subsequently, in 2016, the new broad-spectrum azole isavuconazole (ISA) was approved (Nagy et al. 2021), while itraconazole (ITR) is used for superficial cutaneous and mucosal *Candida* spp. infections (Del Rosso et al. 1998). Nevertheless, treatment of infections associated with biofilm formation is particularly difficult because there are currently no drugs specifically directed against these structures (Wang et al. 2024). Therefore, some antibiotics such as ciprofloxacin (CIP) have been experimentally evaluated because of their ability to interfere with the organization of mixed biofilms or to modify cellular permeability, which could favor the activity of conventional antifungal agents (Manohar et al. 2024).

Given that the development of new classes of antifungals is a slow process, combination therapy emerges as a promising strategy to enhance the efficacy of existing drugs, broaden the spectrum of activity, and prevent the emergence of secondary resistance (Massey et al. 2023). Thus, the combination of antifungal agents with different targets of action (echinocandins, azoles, and polyenes) could exert a synergistic effect capable of destabilizing the complex architecture of biofilms. In this context, the objective of the present study was to evaluate *in vitro* inhibitory activity and synergism of combinations of conventional antifungal

agents against biofilm formation by four *Candida* species, as well as to characterize the alterations induced in their architecture and cellular matrix.

Methods

Antimicrobials

The antifungals of CAS ($\geq 97\%$ purity, HPLC; Sigma-Aldrich, EE. UU., Cat. No. SML0425-5MG) and ISA ($\geq 98\%$ purity, HPLC; Sigma-Aldrich, EE. UU., Cat. No. SML2357-5MG), ITR, AMB and CIP were obtained as clinical-use products (Itraisdin®, ISDIN., 50 mg capsules; Amphotericin B, PiSA®, 50 mg powder for intravenous infusion; Ciproflox®, ALTIA, 400 mg for intravenous infusion). CAS, AMB and CIP were diluted in distilled water, ISA and ITR were diluted in 0.5% DMSO to obtain 1 mM concentration of each antimicrobial.

Microorganisms

The species *C. albicans* (CIMA-306-CER), *C. parapsilosis* (CIMA-307-CER), *C. krusei* (CIMA-308-CER) and *C. tropicalis* (CIMA-309-CER), the first three species are clinical isolates and were donated by the Faculty of Bioanalysis of Universidad Veracruzana. All species were cultured in Sabouraud dextrose broth at 37 °C for 48 h and maintained on Sabouraud dextrose agar at 25 °C. Morphological features were observed on CHROMagar *Candida*®, and microscopic characteristics were determined by crystal violet and safranin staining. In addition, *C. albicans*, *C. krusei*, and *C. parapsilosis* were identified by molecular techniques through polymerase chain reaction (PCR), using ITS1/ITS4 primers from the rDNA ITS regions for amplification and subsequent sequencing. ITS sequences were compared with the NCBI databases (<https://www.ncbi.nlm.nih.gov/>). The BLASTn search tool was used to confirm species identity. The phylogenetic tree was constructed using MEGA 12 software under the GTR + G + I model, and bootstrap support was estimated with 500 replicates (Ejaz et al. 2025). *C. tropicalis* belongs to the Spanish Type Culture Collection (CECT), with identifier CECT1440.

Planktonic susceptibility of *Candida* spp.

The planktonic susceptibility of *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* was determined by the disk diffusion method. Suspensions were adjusted to 0.5 McFarland and seeded on Sabouraud agar plates. Disks containing AMB (20 µg), CAS (10 µg), ITR (10 µg), and ISA (10 µg) were applied, and plates were incubated at 37 ± 2 °C for 24 h (Otto et al. 2023). Inhibition diameters were measured in millimeters and interpreted as susceptible (S) or resistant (R) according to CLSI (2024) breakpoints (Stepanović et al. 2007).

Cell viability was determined using Cell Proliferation Kit II (XTT) (Roche, Cat. No. 11465015001), adding 50 µL of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]. The system was then incubated at 37 °C in the dark for 4 h. Finally, absorbance was measured at 450 and 690 nm to obtain corrected absorbance.

Candida spp. biofilms

The four *Candida* species were precultured on Sabouraud dextrose agar at 27 ± 2 °C. After 48 h, fungal cells were collected and placed in 100 mL of Sabouraud dextrose broth, cultured at 27 ± 2 °C for 24 h, and

processed using the microdilution plate method described by Bisso and colleagues in 2023 (Martínez-Méndez et al. 2023) with some modifications. For this purpose, 200 µL inoculum containing 1×10^8 yeasts/mL (equivalent to tube 0.5 on the McFarland scale) was prepared in 96-well microplates and maintained at 27 ± 2 °C with agitation at 110 rpm for 24 h. After incubation, the supernatant was removed, and the plate was washed four times with phosphate-buffered saline (PBS). The biofilms were then fixed with methanol and stained with 1% Safranin; finally, they were decolorized with 95% ethanol, the excess was removed, and, once dry, absorbance was read in a microplate reader at 450 nm.

The isolates were classified into four categories based on optical density (OD) at 450 nm in relation to the negative-control cutoff value OD_c: non-producer ($OD \leq OD_c$), weak producer ($OD_c < OD \leq 2 \times OD_c$), moderate producer ($2 \times OD_c < OD \leq 4 \times OD_c$), and strong producer $OD > 4 \times OD_c$ (Darwish et al. 2026). Cell viability was determined after biofilm formation and quantification as previously described and according to the manufacturer's instructions for the XTT kit described above. Pharmacological interactions were evaluated by calculating the Fractional Inhibitory Concentration Index (FICI) for biofilms (Zhao et al. 2025).

***In vitro* antifungal activity against mature *Candida* spp. biofilms**

Once mature biofilms (24 h) of the four *Candida* spp. species had formed, they were washed four times with PBS to remove non-adherent cells. The susceptibility of the biofilms was then evaluated both individually and in paired combinations (checkerboard matrix). For the experimental design, serial dilutions of antimicrobials were used within a concentration range selected under a translational approach. Concentrations equivalent to peak plasma and steady-state levels reached in treated patients were included, as well as higher concentrations, considering the resistance of the extracellular biofilm matrix. Concentrations below the standard range were also included to determine the minimum concentration capable of interfering with biofilm stability (Nagy et al. 2021). The specific doses evaluated are detailed in Table 1.

Table 1. Antimicrobial concentrations

Antimicrobial	Concentration (µM)	References
Caspofungin	6.25 – 200	(Nagy et al. 2021)
Isavuconazole	0.5 – 16	(Nagy et al. 2021)
Itraconazole	16.6 – 500	(Abrão et al. 2021)
Amphotericin B	2.5 – 80	(Hacioglu et al. 2020)
Ciprofloxacin	7.5 - 240	(Hacioglu et al. 2020)

Microscopic structure of *Candida* spp. biofilms

The structure and composition of *Candida* spp. biofilms were observed through extracellular polymeric substances (EPS) using a Nikon Eclipse Ti confocal laser scanning microscope (CLSM) equipped with a Nikon C2 module. For the detection of mannose and glucose exopolysaccharides, biofilms were incubated with Concanavalin A conjugated to Alexa Fluor 488 (ConA-AF488) at a final concentration of 5 µg/mL for 24 h in the dark at room temperature. Likewise, to visualize chitin in the cell wall and extracellular matrix, Calcofluor White (0.05 µg/mL) was added (Bojang et al. 2022).

Statistical analysis

Analysis of variance was used to evaluate the effects of the antimicrobials on the planktonic form, as well as their activity against mature biofilms. Tukey's post hoc test was then applied for paired-data analysis. Parametric tests were used to analyze absorbance values for biofilm biomass and metabolic activity when the data showed symmetry and similar variances. For non-parametric data, the Friedman test was used, followed by Dunn's post hoc test. Statistical analysis was performed with GraphPad Prism® 10.0 (GraphPad Software, San Diego, CA, USA).

Results

Microorganisms

The sequences obtained with the ITS1 and ITS4 were used to confirm the taxonomic identity of the isolates through a Maximum Likelihood phylogenetic analysis (Fig. 1), in which the isolates clustered into monophyletic clades with their respective reference strains. Likewise, the samples cultured on CHROMagar™ showed species-specific chromatic characteristics (Fig. 2), thus *C. krusei* (a) presented pink colonies and rod-shaped cells accompanied by hyphae and pseudohyphae, whereas *C. albicans* (b) exhibited bright green colonies composed of round yeasts, *C. parapsilosis* (c) developed cream-colored colonies consisting of round yeasts and pseudohyphae, while *C. tropicalis* (d) was distinguished by metallic blue colonies with elongated yeasts and pseudohyphae.

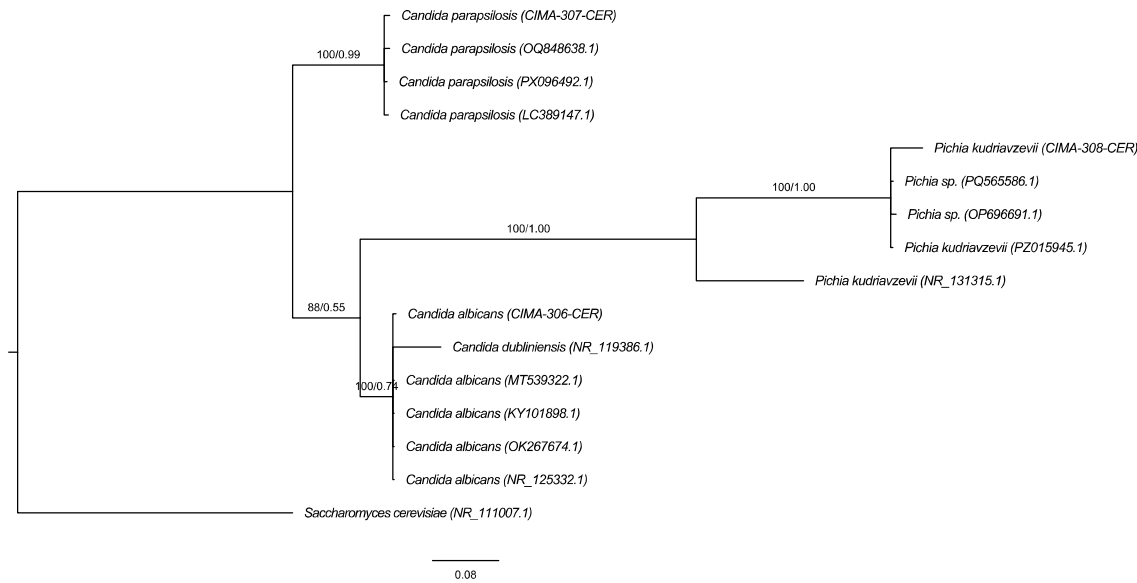


Fig. 1 Maximum Likelihood phylogeny for *Candida* spp. Branch support values (bootstrap) were obtained after 1,000 replicates and are shown at the corresponding nodes; the tree was rooted with the outgroup species *Saccharomyces cerevisiae*. Maximum-likelihood bootstrap values (BS) >50% and Bayesian posterior probabilities (BPP) >0.75. Note: *C. krusei* (now *Pichia kudriavzevii*)

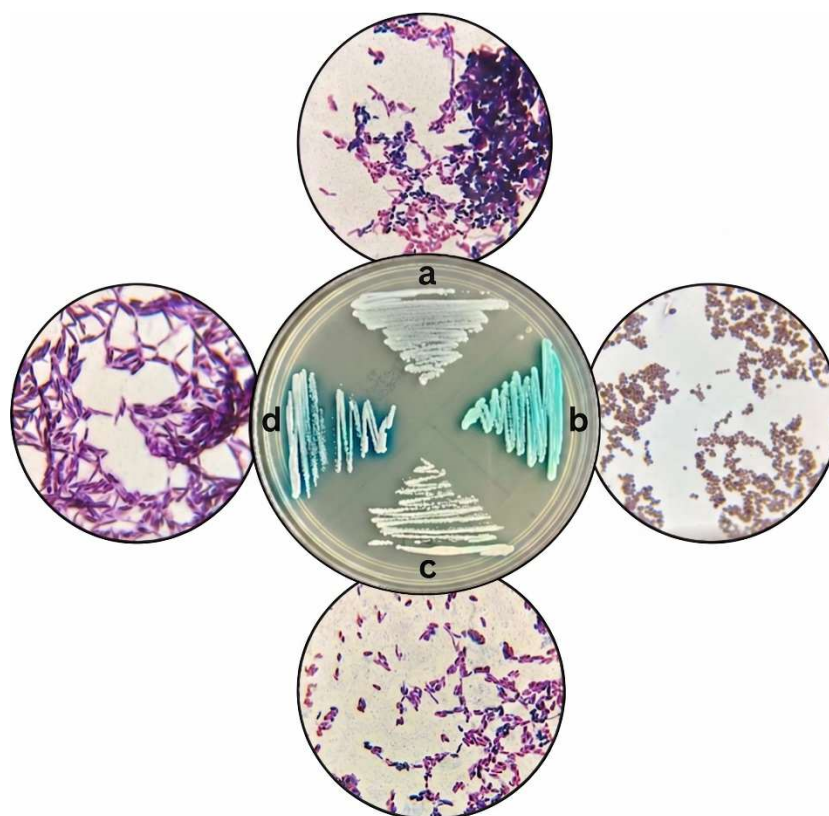


Fig. 2 Morphological identification of *Candida* species. Central culture on CHROMagar™ chromogenic medium and corresponding peripheral microphotographs (Gram staining, optical microscopy 100X). **a)** *C. krusei* (pink colonies); round yeasts. **b)** *C. albicans* (green colonies); rod-shaped yeasts with hyphae and pseudohyphae. **c)** *C. parapsilosis* (cream-colored colonies); elongated yeasts and pseudohyphae. **d)** *C. tropicalis* (metallic-blue colonies); round yeasts and pseudohyphae

Planktonic susceptibility of *Candida* spp.

The results of the antifungal susceptibility testing (Fig. 3) showed a species-dependent response, overall, based on the zones of inhibition *C. albicans* was classified as susceptible to CAS, with a 17 mm zone of inhibition; in contrast, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* were resistant, with zones of inhibition of 15, 9 and 13 mm, respectively. For ISA, *C. albicans* was the only susceptible species (13 mm), while the others were reported as resistant. Regarding ITR, susceptibility was observed in *C. albicans* (13 mm) and *C. tropicalis* (16 mm), whereas *C. albicans* and *C. parapsilosis* displayed resistance, with zones of inhibition of 2 and 9 mm. AMB only managed to inhibit *C. tropicalis* (15 mm), with the remaining species classified as resistant. Finally, none of the evaluated species showed susceptibility to ciprofloxacin; all were resistant.

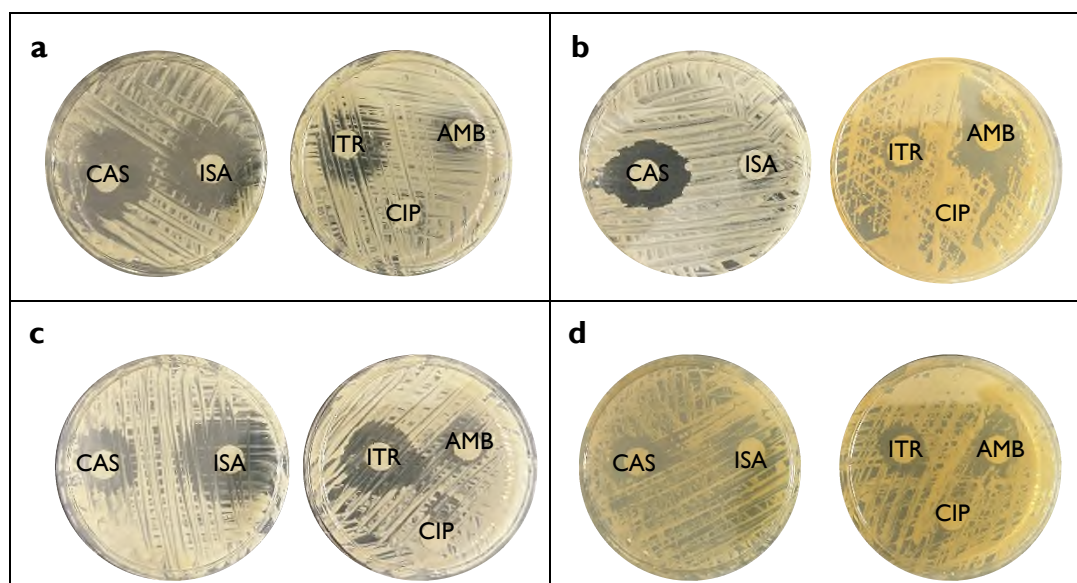


Fig. 3 Antifungal susceptibility profiles of *Candida* spp. *C. albicans* (a), *C. krusei* (b), *C. tropicalis* (c) and *C. parapsilosis* (d). Where: Caspofungin (CAS), Isavuconazole (ISA), Itraconazole (ITR), Amphotericin B (AMB) y Ciprofloxacin (CIP)

Planktonic susceptibility was determined through an XTT cell-viability analysis (Fig. 4) based on these results, it is evident that CAS, both individually and in combination, exerts significant inhibitory activity against the four species of *Candida* evaluated, with a response that varies markedly according to species and treatment concentration. In the untreated control group, all species exhibited homogeneous and maximal viability, which methodologically validates the experiments because no initial statistical differences were recorded among them. Analysis of CAS as a single compound (Fig. 4a) revealed a highly marked differential susceptibility profile, *C. albicans* was the species with the greatest basal resistance, maintaining cell viability levels above 50% even at the highest concentration of 50 μ M, however, the non-*albicans* species showed considerably greater susceptibility; in particular, *C. tropicalis* and *C. parapsilosis* underwent a drastic reduction in viability from the lowest dose of 12.5 μ M, stabilizing at approximately 25% without major changes as concentration increased. This suggests that maximal metabolic inhibition was reached at the initial thresholds. The response to AMB alone (Fig. 4b) confirmed a strong generalized antifungal activity, highlighting that *C. albicans* appears to reach an inhibition plateau between 10 and 20 μ M, where residual viability remained practically unchanged. Both CAS + ISA (Fig. 4c) and CAS + AMB (Fig. 4d) enhanced the overall antifungal effect. This was especially evident in *C. albicans*: while this pathogen effectively resisted the maximum concentrations of each compound when used individually, combined exposure at the highest doses (50 + 4 μ M and 50 + 20 μ M, respectively) overcame this resistance and reduced viability in a statistically significant manner compared with single-drug treatments. Analysis of variance and Tukey's test corroborated the robustness of these observations: the higher tolerance of *C. albicans*, the intermediate susceptibility of *C. krusei*, and the high sensitivity of *C. tropicalis* and *C. parapsilosis* corresponded to biologically meaningful differences with statistical significance ($p < 0.05$).

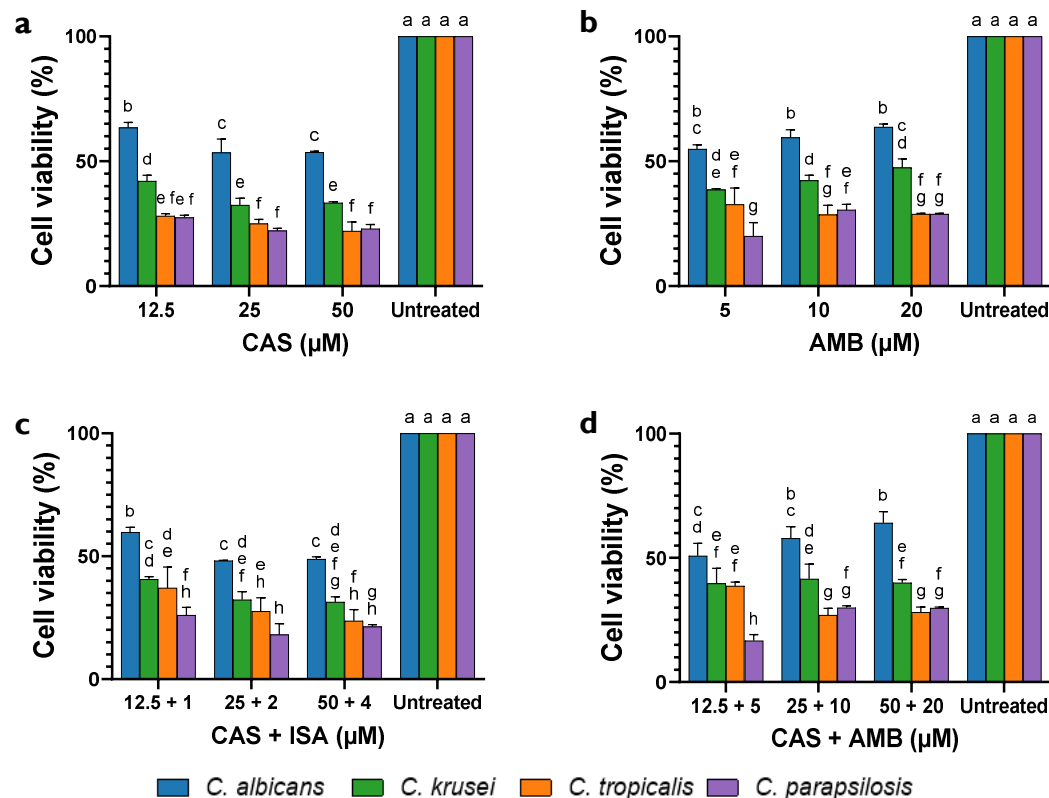


Fig. 4 Cell viability of planktonic state of *Candida* spp. Bars represent mean absorbance values (450–690 nm) $\pm$ SD compared with untreated control biofilms. Significant differences ($p < 0.05$) are indicated by different letters (ANOVA, Tukey's test)

Candida spp. biofilms

All evaluated strains demonstrated the ability to form biofilms, although with variable intensity. Biomass assays classified *C. albicans* and *C. parapsilosis* as strong producers, characterized by a dense matrix and high adhesion to the polystyrene surface. By contrast, *C. tropicalis* and *C. krusei* behaved as moderate producers under the experimental conditions tested.

Assays performed on mature biofilms revealed a significant decrease in susceptibility to all antifungal agents compared with planktonic cells. Biofilms of *C. albicans* and *C. parapsilosis* retained some sensitivity to combinations that included CAS, whereas *C. krusei* and *C. tropicalis* showed high resistance even under combined treatments.

Heatmaps (Fig. 5 and Fig. 6) enabled comparative visualization of biofilm susceptibility patterns under individual and combined treatments. In these maps, lighter shades indicate greater growth inhibition and biomass reduction, whereas darker shades reflect resistance or a poor response to treatment. About single-drug application (Fig. 5), CAS was the antifungal agent with the greatest antibiofilm capacity, showing activity against the evaluated species except *C. tropicalis*. For the remaining antimicrobials tested individually, biofilm formation appeared to persist, even if only slightly.

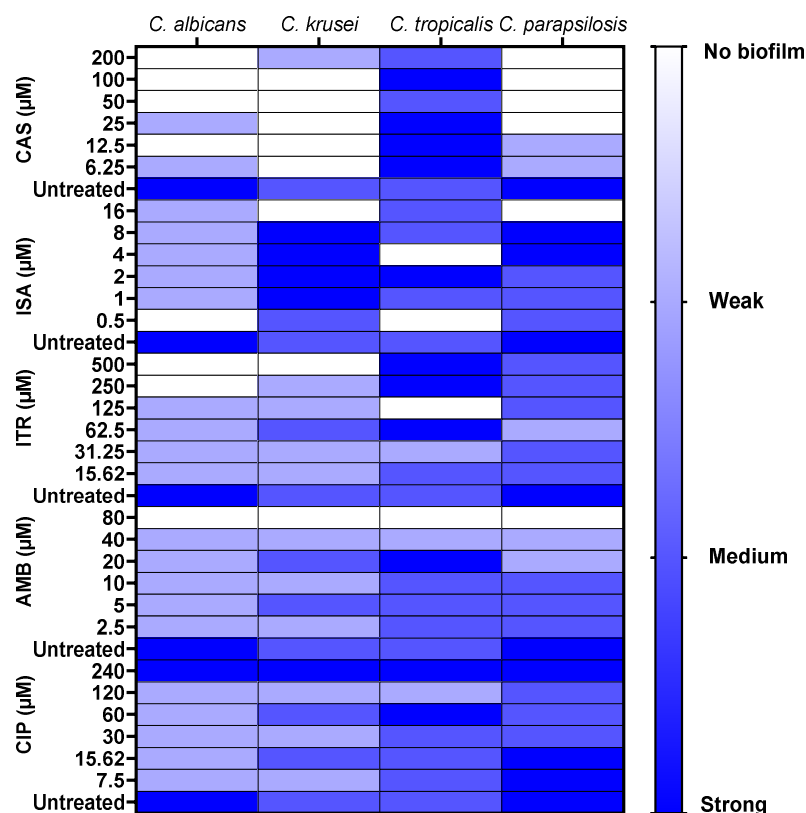


Fig. 5 Heatmap for biofilm-forming capacity of *Candida* species under individual antimicrobial treatments. Heatmap displaying the classification of biofilm production for *C. albicans*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* exposed to varying concentrations (μM) of caspofungin (CAS), isavuconazole (ISA), itraconazole (ITR), amphotericin B (AMB), and ciprofloxacin (CIP). White cells indicate no biofilm formation; dark blue cells indicate strong biofilm formation

In the antimicrobial-combination analysis (Fig. 6), the antibiofilm capacity of CAS + ISA and CAS + AMB was particularly evident. It was also observed that monotherapies required high concentrations to achieve detectable inhibition, however, combining CAS + ISA or AMB markedly reduced the doses required to compromise the fungal structure. Based on the fractional inhibitory concentration index (FICI) (Table 2), in *C. albicans* the CAS + ISA combination showed synergy (FICI = 0.5), whereas combinations with AMB exhibited antagonism (FICI > 4.0), for *C. krusei*, CAS combinations resulted in indifferent interactions (FICI ≈ 1.03), in *C. parapsilosis*, all evaluable combinations showed indifference (FICI between 1.06 and 3.00), in *C. tropicalis*, values could not be determined (-) because of the absence of individual inhibition, although biofilm reduction was observed in heatmaps with CAS + ISA and CAS + AMB, suggesting that the pharmacological interaction varies drastically by species. Although FICI values did not strictly meet the mathematical criteria for synergy in all species, the drug combinations exerted a greater impact on biofilm viability. These findings justify the need for complementary visual analysis.

Table 2. Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration indices (FICI) of antimicrobial agents, alone and combination, against *Candida* species biofilms.

Species	MIC (μM)				MIC [drug A+drug B (μM)] (FICI)		
	CAS	ISA	ITR	AMB	CAS+ISA	CAS+ITR	CAS+AMB
<i>C. albicans</i>	12.5	0.5	250	80	6.25 + 0.5 ^s	200 + 500 ^A	200 + 80 ^A
<i>C. krusei</i>	6.25	16	500	80	6.25 + 0.5 ^I	6.25 + 15.62 ^I	200 + 80 ^A
<i>C. tropicalis</i>	-	0.5	125	2.5	-	-	6.25 + 2.5
<i>C. parapsilosis</i>	12.5	16	-	2.5	12.5 + 1.0 ^I	12.5 + 31.25	12.5 + 5 ^I

Note: MIC values are expressed in μM Abbreviations; CAS, caspofungin; ISA, isavuconazole; ITR, itraconazole; AMB, amphotericin B. Symbol (-), no inhibition at the highest concentration tested; FICI Interpretation:

^s Synergy: ≤ 0.5

^I Indifference: >0.5 – 4.0

^A Antagonism: >4.0

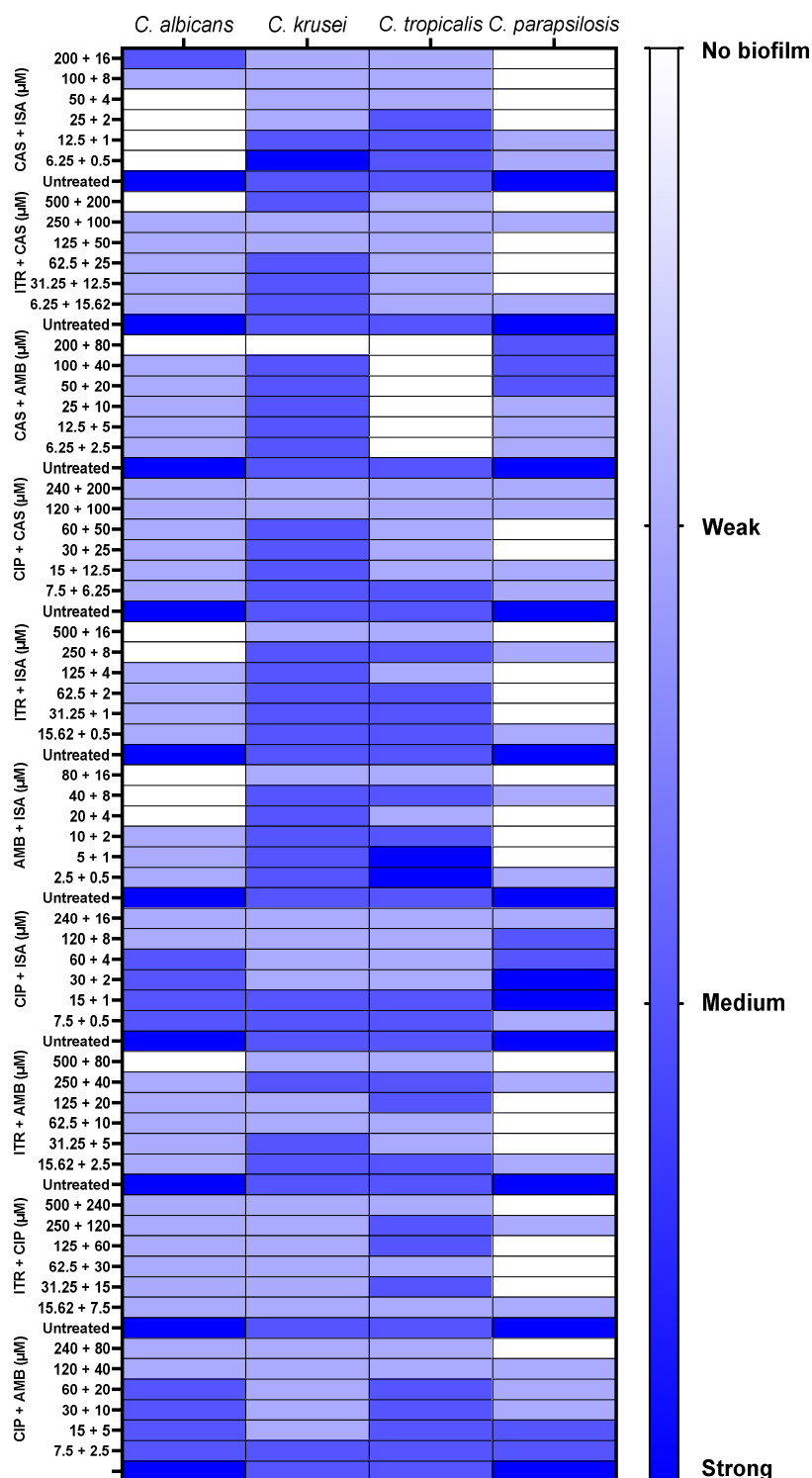


Fig. 6 Heatmap for biofilm-forming capacity of *Candida* species treated with antimicrobial combinations. Heatmap illustrates biofilm production levels under pairwise combinations of caspofungin (CAS), isavuconazole (ISA), itraconazole (ITR), amphotericin B (AMB), and ciprofloxacin (CIP). The vertical axis indicates the specific concentration (μM) of the combined agents. The color gradient corresponds to the biofilm classification defined in figure 1: 0 (white, non-producer) to 3 (dark blue, strong producer)

Cell viability in mature biofilms was evaluated because reduction or inhibition of biofilm formation does not necessarily guarantee death of *Candida* spp. yeasts (Fig. 7). This analysis was performed to determine whether the observed resistance or susceptibility in biofilms was due to intrinsic genetic mechanisms of the yeasts or to protection conferred by the extracellular polymeric substance matrix characteristic of biofilms. *C. albicans* was found to retain the highest levels of metabolic activity, between 50% and 70%, preserving cell viability even under the pressure of combined therapies. This species, together with *C. krusei* and *C. parapsilosis*, showed susceptibility to the CAS + ISA combination. In the specific case of *C. tropicalis*, although a reduction in metabolic activity was observed with CAS + ISA, the CAS + AMB combination produced a notable reduction; metabolic activity levels were significantly greater than those obtained with any other treatment.

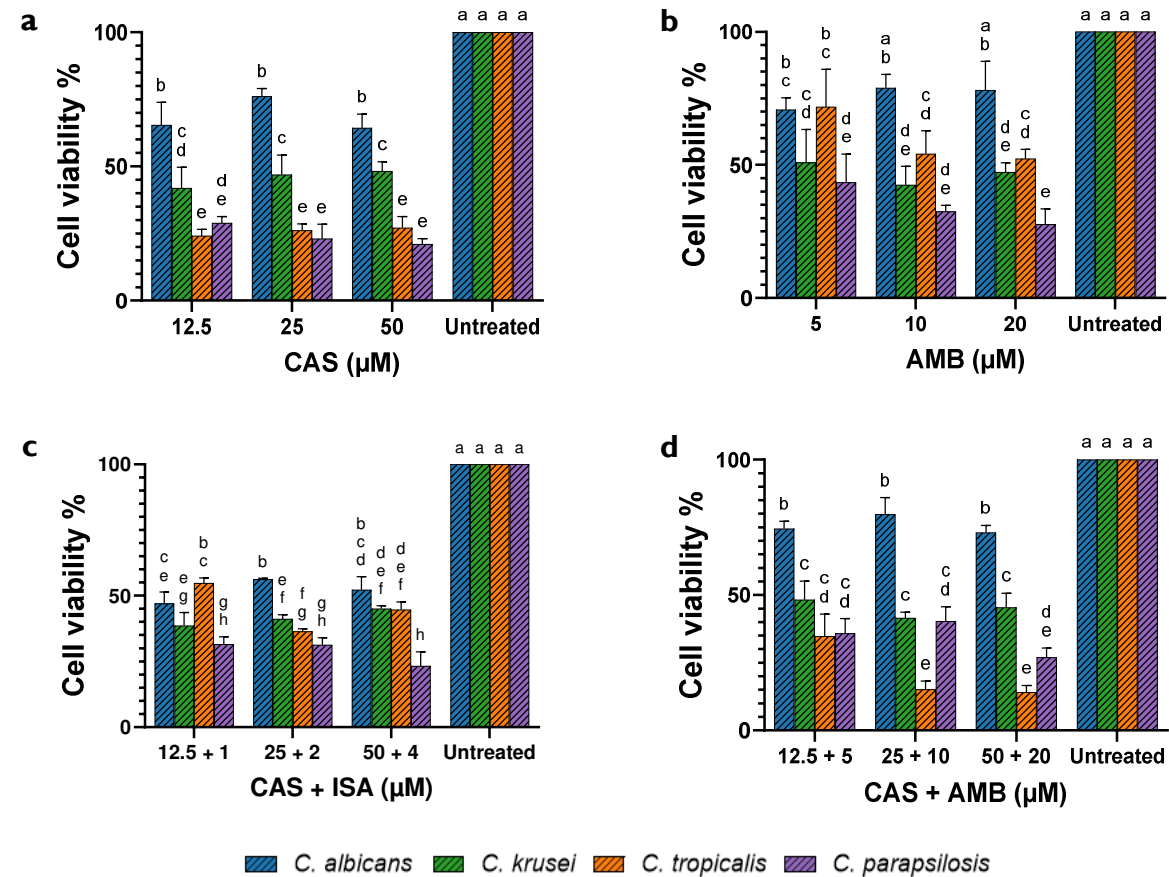


Fig. 7 Cell viability of *Candida* spp. Biofilms. Bars represent mean absorbance values (450–690 nm) ± SD compared with untreated control biofilms. Significant differences ($p < 0.05$) are indicated by different letters (ANOVA, Tukey's test)

Microscopic structure of *Candida* spp. biofilms

Confocal microscopy analysis (Fig. 8 and Fig. 9) enabled observation of the three-dimensional architecture and distribution of extracellular matrix components in the biofilms. Biofilms were stained with Concanavalin A conjugated to Alexa Fluor 488 (green), which identifies mannose- and glucose-rich

exopolysaccharides, and with Calcofluor White (blue), which labels chitin present in the cell wall and matrix. In *C. albicans*, the control biofilm showed a dense and homogeneous structure with an abundant matrix. In *C. krusei*, the biofilm showed a less compact and more irregular organization; antifungal treatment partially reduced the fluorescence signal, but areas containing intact cells persisted, suggesting lower structural susceptibility, in *C. parapsilosis*, the reduction in green and blue fluorescence after antifungal exposure was evident, indicating a significant alteration in matrix composition and cellular adhesion, finally, in *C. tropicalis* after treatment, fluorescence was markedly reduced, reflecting a loss of matrix continuity and a decrease in biofilm thickness (Fig. 8).

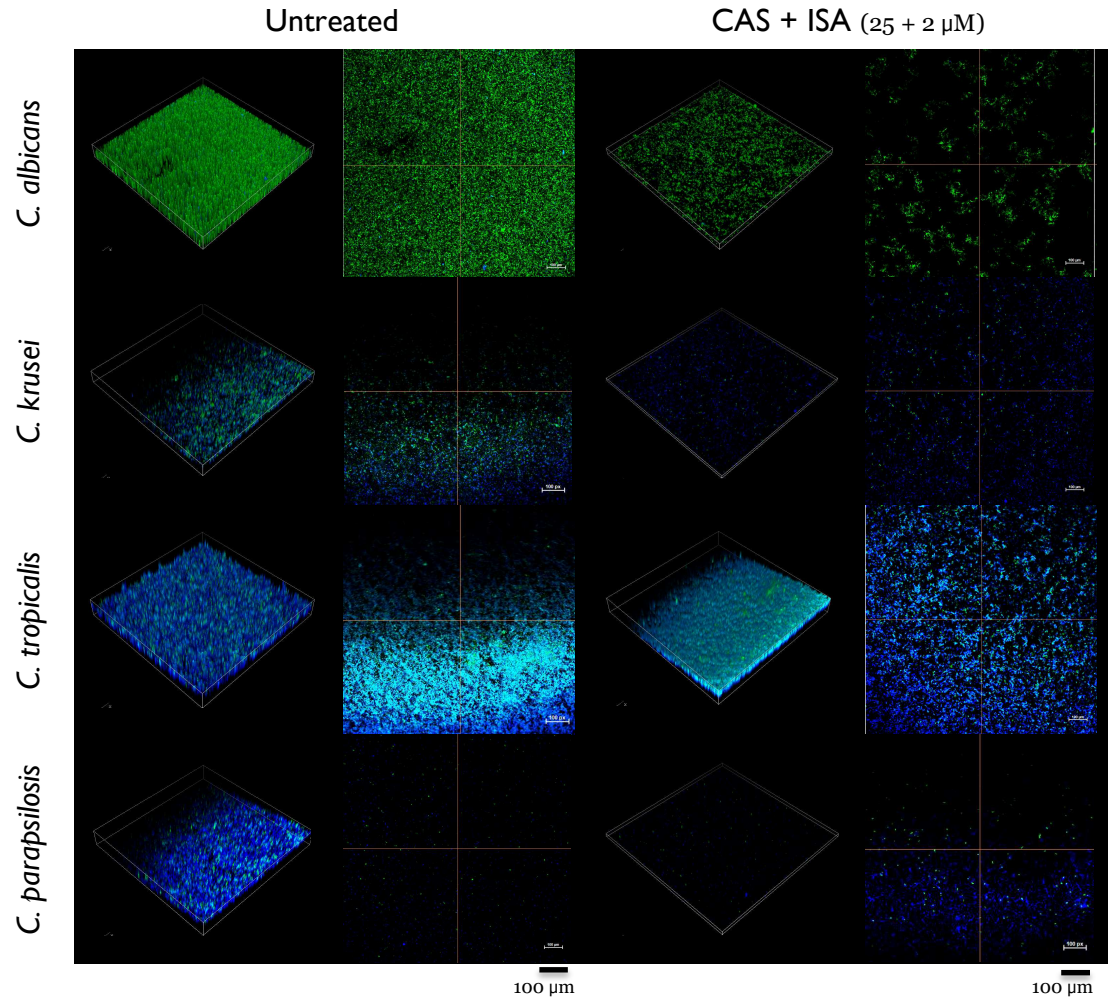


Fig. 8 Spatial distribution of exopolysaccharide components in *Candida* spp. biofilms. Representative micrographs show the distribution of exopolysaccharides (green, stained with Concanavalin A–Alexa Fluor 488) and chitin (blue, stained with Calcofluor White)

Table 3 shows the mechanism of action of the CAS + ISA combination on biofilm structure, a reduction in both cell viability and the EPS matrix was observed, in *C. albicans*, reduction of both components was

moderate (50%), whereas in *C. tropicalis* and *C. parapsilosis*, the treatment achieved an almost complete reduction of the extracellular matrix, decreasing EPS to levels below 20%. These results indicate that, although a fraction of the cell population remains viable, the structural integrity of the biofilms is compromised by the treatment.

Table 3. Quantitative biofilm parameters in *Candida* species under treatment with CAS + ISA (25 + 2 μ M)

Species	Evaluated Parameter	Untreated	CAS+ISA (25 + 2 μ M)	Change / Reduction	p-value
<i>C. albicans</i>	Average Thickness (μ m)	33.17 $\pm$ 3.20	30.17 $\pm$ 7.22	↓ 9.04%	ns
	Chitin (%)	100	47.34 $\pm$ 27.34	↓ 52.66%	<0.01 (**)
	EPS (%)	100	62.62 $\pm$ 30.85	↓ 37.38%	<0.01 (**)
<i>C. krusei</i>	Average Thickness (μ m)	52.00 $\pm$ 5.15	55.61 $\pm$ 19.97	+ 6.94%	ns
	Chitin (%)	100	51.72 $\pm$ 32.76	↓ 48.28%	<0.001 (****)
	EPS (%)	100	25.15 $\pm$ 20.42	↓ 74.85%	<0.001 (****)
<i>C. tropicalis</i>	Average Thickness (μ m)	45.06 $\pm$ 4.80	51.33 $\pm$ 16.50	+ 13.91%	ns
	Chitin (%)	100	46.23 $\pm$ 16.39	↓ 53.77%	<0.001 (****)
	EPS (%)	100	10.16 $\pm$ 15.72	↓ 89.84%	<0.001 (****)
<i>C. parapsilosis</i>	Average Thickness (μ m)	47.67 $\pm$ 4.50	48.06 $\pm$ 7.11	+ 0.82%	ns
	Chitin (%)	100	38.17 $\pm$ 25.08	↓ 61.83%	<0.001 (****)
	EPS (%)	100	16.90 $\pm$ 4.96	↓ 83.10%	<0.001 (****)

Values are expressed as mean $\pm$ standard deviation (SD). Chitin and extracellular polymeric substances (EPS) data are normalized relative to the untreated control group (100%). Statistically significant differences compared to the respective untreated control are indicated by asterisks: **p < 0.01, ****p < 0.0001; ns: non-significant difference (p > 0.05) determined by Two-way ANOVA with Tukey's post-hoc multiple comparisons test.

In the case of *C. tropicalis* the CAS + AMB combination (Fig. 9) caused a marked decrease in fluorescence and thinning of the biofilm compared with the control. The matrix appeared less dense and fragmented, indicating considerable structural damage and loss of cohesion (Table 4). Overall, the confocal observations confirm the metabolic-activity results, demonstrating that antifungal combinations, especially those including caspofungin, induce pronounced disorganization in biofilm structure, although the magnitude of the effect differs among species.

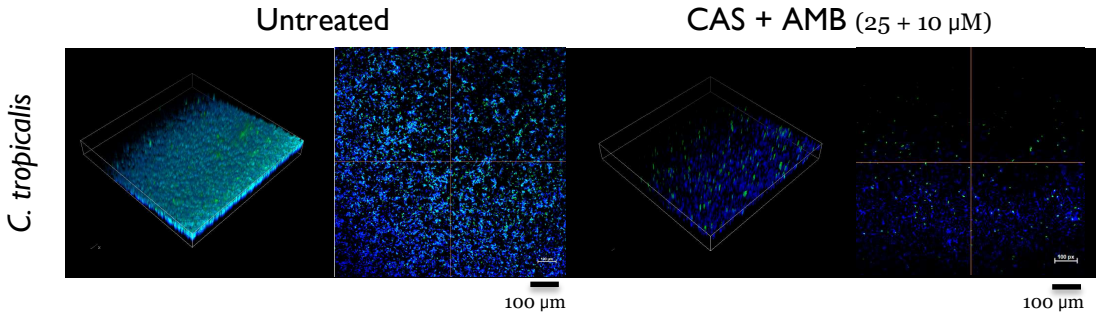


Fig. 9 Spatial distribution of exopolysaccharide components in *Candida tropicalis* biofilms. Representative micrographs show the distribution of exopolysaccharides (green, stained with Concanavalin A–Alexa Fluor 488) and chitin (blue, stained with Calcofluor White)

Table 4. Quantitative biofilm parameters in *Candida tropicalis* under treatment with CAS + AMB (25 + 10 µM)

Species	Evaluated Parameter	Untreated	CAS + AMB (25 + 10 µM)	Change / Reduction	p-value
<i>C. tropicalis</i>	Average Thickness (µm)	46.06 ± 3.80	51.04 ± 22.14	+ 13.27 %	ns
	Chitin (%)	100	37.07 ± 21.03	↓ 62.93%	<0.0001 (****)
	EPS (%)	100	43.91 ± 38.07	↓ 56.09%	<0.0001 (****)

Values are expressed as mean ± standard deviation (SD). Chitin and extracellular polymeric substances (EPS) data are normalized relative to the untreated control group (100%). Statistically significant differences compared to the respective untreated control are indicated by asterisks: **p < 0.01, ****p < 0.0001; ns: non-significant difference (p > 0.05) determined by Two-way ANOVA with Tukey's post-hoc multiple comparisons test.

Discussion

The emergence of non-*albicans* *Candida* species and the increasing prevalence of biofilm-associated infections represent one of the greatest challenges in contemporary medical mycology. Fungal biofilms act as reservoirs of persistent infection, conferring on yeasts an intrinsic resistance to antifungal agents that may be up to 1,000 times greater than that of their planktonic counterparts (Hoenigl et al. 2021; Wang et al. 2024). This resistance is exacerbated by the current global antimicrobial-resistance crisis, in which the extracellular matrix acts not only as a physical barrier but also as a chemical one, limiting drug bioavailability and allowing sessile cells to survive even at concentrations that would be lethal in the free-living state (Giono-Cerezo et al. 2020).

The World Health Organization has recently classified *C. tropicalis* and *C. krusei* as high- and medium-priority pathogens, respectively (Zhao et al. 2025), our planktonic susceptibility results corroborate the multidrug-resistance profile of *C. krusei*, consistent with global reports warning of its poor response to conventional azoles (Tan et al. 2021). However, the most significant finding of this investigation lies in the activity of the combinations against *C. tropicalis* biofilms. Although this species behaved as a moderate biofilm producer under our experimental conditions, its matrix proved highly persistent. It should be noted that, although molecular tools have led to the reclassification of some species formerly belonging to the genus *Candida* the mycoses caused by them continue to be considered candidiasis; this applies to *C. krusei* now *Pichia kudriavzevii* (Nguyen et al. 2024).

This study highlights that combining antifungals with complementary targets of action (cell wall and plasma membrane) is a necessary strategy for destabilizing mature biofilms, although the biological response varies drastically among species (Fan et al. 2022), *C. albicans* biofilms manifested as resistant structures, retaining the highest levels of residual metabolic activity even after combined treatment with CAS and ISA. This observation suggests that CAS and ISA, *C. albicans* specific resistance mechanisms, such as high extracellular-matrix density and the presence of persistent cells, mitigate the damage generated by caspofungin on β-(1,3)-D-glucan, allowing a significant fraction of the community to maintain biological functionality.

Microscopic observations by Confocal Laser Scanning Microscopy (CLSM) suggest that caspofungin plays an 'opening' role in combination therapy. Caspofungin weakens the architecture of the extracellular matrix (Nagy et al. 2021), visualized in this study as a significant reduction in Concanavalin A fluorescence. This matrix disruption reduces the physical barriers that normally sequester antifungals, theoretically allowing greater intracellular penetration of isavuconazole (which inhibits ergosterol synthesis) or amphotericin B

(which binds directly to membrane ergosterol) (Ajetunmbi et al. 2023). This pharmacodynamic synergy, in which the cell wall and cell membrane are attacked simultaneously, is critical for eradicating mature biofilms, where cells persist and the polymeric matrix limits the efficacy of monotherapy (Lee et al. 2021). The efficacy observed in this study for the combination of caspofungin and isavuconazole against mature biofilms is consistent with previous reports in species of high clinical criticality. In this regard, Nagy et al. (2021) demonstrated that this combination exhibits potent *in vitro* synergism against *Candida auris* (now *Candidozyma auris*), reducing isavuconazole concentrations by up to 512-fold. The findings obtained reinforce the premise that dual targeting (cell wall and membrane) is not only effective against species such as *C. auris* but also constitutes a strategy for destabilizing extracellular-matrix architecture in other species such as *C. albicans* and non-*albicans* species, including *C. krusei* and *C. tropicalis*, in which monotherapies often fail because of high sessile inhibitory concentrations.

A phenomenon of biological interest observed in *C. tropicalis* was the significant increase in biofilm thickness after combined treatment, despite the massive cell death detected by the XTT assay. This may be due to activation of stress-response mechanisms in the fungal cell wall, possibly mediated by the calcineurin pathway or the cell-wall integrity pathway, which induce overcompensatory synthesis of chitin and other structural polymers in response to glucan inhibition (Malinovská et al. 2023). CLSM images confirmed this hypothesis, showing an intense Calcofluor White signal (a chitin marker) in treated biofilms. This finding emphasizes that biomass reduction should not be the only indicator of therapeutic success and that stress may represent a failed-survival phenotype under strong dual blockade.

It is important to emphasize that, in *C. albicans* and *C. parapsilosis*, although calculation of the Fractional Inhibitory Concentration Index (FICI) yielded indifference values for the CAS + ISA combination, microscopic and metabolic evaluation revealed substantial biological damage. As noted by Nagy et al. 2024, *in vitro* interactions with echinocandins often do not meet the strict criteria for FICI-defined synergy because of the fungistatic nature of azoles and the paradoxical effect; however, this does not rule out their clinical usefulness for reducing fungal burden and destabilizing biofilms *in vivo*. Conversely, the antagonism observed between caspofungin and amphotericin B in *C. albicans* highlights the need to characterize species-specific responses before implementing empirical combination therapies (Prusty et al. 2025).

In conclusion, this study demonstrates that combining caspofungin with isavuconazole or caspofungin with amphotericin B constitutes a promising strategy that overcomes the limitations of current monotherapy. Specifically, the CAS + ISA combination proved highly effective in altering the biofilm matrix and reducing cell viability in *Candida* spp. Similarly, CAS + AMB affected *C. tropicalis*, despite inducing a stress-related structural thickening response. Although monotherapy against mature biofilms has significant limitations, the strategic combination of echinocandins with azoles or polyenes compromises the structural integrity of these communities, with particularly notable efficacy against *C. tropicalis*. Before evaluating these combinations, ciprofloxacin (CIP) was included in the experimental design because of its ability to interfere with biofilm organization or modify cellular permeability, which could theoretically favor the action of conventional antifungal agents (Sass et al. 2022), however, FICI analysis showed that its effect is limited against mature fungal biofilms and in the planktonic state.

Although CAS reduces viability compared with the control, residual absorbance levels residual in the four species indicate that a considerable fraction of the sessile population remains metabolically active, especially in *C. albicans* and *C. krusei*, the use of CAS alone permits the persistence of communities that retain substantial XTT-reduction capacity. This suggests that, although CAS damages the cell wall, it cannot neutralize the internal metabolism of sessile cells when used alone. CAS monotherapy therefore carries the risk of an incomplete response in structures matured for 24 h. When combined with ISA, the cell is attacked at two critical points simultaneously: structural integrity (cell wall) and membrane function (ergosterol). The results show that, even if the CAS-alone and CAS + ISA bars present similar values, the combination ensures a more comprehensive metabolic blockade, preventing the yeast from using compensatory pathways that could be activated by cell-wall stress alone. These data support the hypothesis that simultaneous targeting of the cell wall and cell membrane destabilizes the biofilm and decreases cellular proliferation in *Candida* spp. In this way, the study lays the groundwork for a potential therapeutic alternative for persistent infections caused by emerging non-albicans species classified as high- and medium-priority fungal pathogens. Future in vivo studies are required to validate whether this structural disruption translates into clinical improvement in patients with invasive candidiasis.

Statements and Declarations

Author Contributions

Conceptualization, A.C. and C.E.; methodology, A.C., F.C.C., and A.R.M.; formal analysis C.E. and A.C.; investigation C.E., A.C., and F.C.C.; data curation A.C. and F.C.C.; writing—original draft preparation A.C. and F.C.C.; writing—review and editing A.C. and C.E., supervision, A.C. and A.R.M.; project administration, C.E. and A.C.; and funding acquisition, C.E. All authors have read and agreed to the published version of the manuscript.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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