



# Caspofungin Inhibits Mixed Biofilms of *Candida albicans* and Methicillin-Resistant *Staphylococcus aureus* and Displays Effectiveness in Coinfected *Galleria mellonella* Larvae

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**ABSTRACT** *Candida albicans* and *Staphylococcus aureus* are pathogens commonly isolated from bloodstream infections worldwide. While coinfection by both pathogens is associated with mixed biofilms and more severe clinical manifestations, due to the combined expression of virulence and resistance factors, effective treatments remain a challenge. In this study, we evaluated the activity of echinocandins, especially caspofungin, against mixed biofilms of *C. albicans* and methicillin-resistant (MRSA) or methicillin-susceptible *S. aureus* (MSSA) and their effectiveness *in vivo* using the *Galleria mellonella* coinfection model. Although caspofungin (CAS) and micafungin (MFG) inhibited the mixed biofilm formation, with CAS exhibiting inhibitory activity at lower concentrations, only CAS was active against preformed mixed biofilms. CAS significantly decreased the total biomass of mixed biofilms at concentrations of  $\geq 2 \mu\text{g/ml}$ , whereas the microbial viability was reduced at high concentrations (32 to 128  $\mu\text{g/ml}$ ), leading to fungus and bacterium cell wall disruption and fungal cell enlargement. Notably, CAS (20 or 50 mg/kg of body weight) treatment led to an increased survival and improved outcomes of *G. mellonella* larvae coinfecting with *C. albicans* and MRSA, since a significant reduction of fungal and bacterial burden in larval tissues was achieved with induction of granuloma formation. Our results reveal that CAS can be a therapeutic option for the treatment of mixed infections caused by *C. albicans* and *S. aureus*, supporting additional investigation.

**IMPORTANCE** Infections by microorganisms resistant to antimicrobials is a major challenge that leads to high morbidity and mortality rates and increased time and cost with hospitalization. It was estimated that 27 to 56% of bloodstream infections by *C. albicans* are polymicrobial, with *S. aureus* being one of the microorganisms commonly coisolated worldwide. About 80% of infections are associated with biofilms by single or mixed species that can be formed on invasive medical devices, e.g., catheter, and are considered a dissemination source. The increased resistance to antimicrobials in bacterial and fungal cells when they are in biofilms is the most medically relevant behavior that frequently results in therapeutic failure. Although there are several studies evaluating treatments for polymicrobial infections associated or not with biofilms, there is still no consensus on an effective antimicrobial therapy to combat the coinfection by bacteria and fungi.

**KEYWORDS** antibiofilm, polymicrobial infection, echinocandin, micafungin, resistance, MRSA

Polymicrobial infections caused by bacteria and fungi are recognized with increasing frequency in medical settings (1). In this regard, *Candida albicans* and *Staphylococcus aureus* are the most common fungal and bacterial pathogens isolated from bloodstream coinfections worldwide (2, 3). Worryingly, coinfections have clinical implications, limiting

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therapeutic options, especially when they involve the presence of multidrug-resistant lineages (e.g., methicillin-resistant *Staphylococcus aureus*, MRSA) and biofilm formation, often associated with higher mortality rates (2, 4–6).

Biofilms are heterogeneous microbial communities attached to biotic or abiotic surfaces, including catheters, and indwelling medical devices that act as infection and dissemination sources. Involved in an extracellular matrix (ECM), biofilms form three-dimensional (3D) structures, where cells have an altered phenotype that differs from their planktonic counterpart, mainly in relation to the reduction of the antimicrobial susceptibility, a key feature that impacts the persistence of infection and contributes to therapeutic failure (7, 8).

While echinocandins (anidulafungin, caspofungin, and micafungin) have been first-line antifungal agents recommended for the treatment of biofilm-associated *Candida* infection (9), effective therapies for established staphylococcal biofilms are not available yet (8, 10). Moreover, there are few studies on treatment strategies against polymicrobial biofilms of *C. albicans* and *S. aureus* (10, 11). Therefore, combating single/mixed biofilms is considered a challenge for both researchers and clinicians.

It is worth noting that caspofungin and other echinocandins, combined or not with antibacterial compounds, have shown inhibitory activity against planktonic cells and biofilms of Gram-positive and Gram-negative pathogens (12–17). In fact, previous studies have shown homology of *N*-acetylglucosamine transferase enzymes from *S. aureus* to  $\beta$ -1,3-glucan synthase enzymes from *C. albicans* (12). Therefore, in this study, we have evaluated the echinocandin activity, especially caspofungin, against mixed biofilms of *C. albicans* and methicillin-susceptible *S. aureus* (MSSA) or MRSA, and the efficacy in the treatment of coinfecting *Galleria mellonella* larvae.

## RESULTS

**Activity of antimicrobials on planktonic cells.** While *S. aureus* ATCC 29213 and ATCC 6538 were susceptible to all tested antibacterials, *S. aureus* ATCC 33591 was resistant to all antibacterial agents, except to trimethoprim, confirming a MRSA phenotype (see Table S1 in the supplemental material). Caspofungin (CAS) and micafungin (MFG) inhibited the planktonic cells of *C. albicans* SC5314 and IAL-40, displaying fungicidal activity, whereas only CAS showed inhibitory and bactericidal activities against *S. aureus* planktonic cells (Table S2).

**Caspofungin inhibits the mixed biofilm formation and preformed biofilm by *Candida albicans* and *Staphylococcus aureus*.** CAS and MFG reduced *C. albicans* biofilms, both in formation and 24 h preformed. *S. aureus* biofilms were susceptible to vancomycin (VCM) and both echinocandins. In this regard, CAS inhibited the biofilm formation at lower concentrations than MFG, but only CAS was able to reduce preformed bacterial biofilms (Table S3).

Mixed biofilms formed by *C. albicans* IAL-40 and *S. aureus* were more susceptible to echinocandins and VCM (Table 1), most likely due to the lower ability of the IAL-40 strain to form a robust mixed biofilm compared to those formed by the SC5314 strain (Fig. S1). In fact, *C. albicans* SC5314/*S. aureus* biofilms were not susceptible to VCM, whereas CAS was slightly more active than MFG against biofilm in formation and the only agent active against 24-h-preformed biofilms (Table 1). Thus, a deeper antibiofilm analysis was performed using CAS, *C. albicans* SC5314, and *S. aureus* strains.

**Caspofungin reduces the biomass and cell viability of mixed biofilms of *Candida albicans* and *Staphylococcus aureus*.** CAS treatment resulted in a similar antibiofilm effect against the mixed and single biofilms (Fig. 1, Fig. S2). In this regard, while CAS at  $\geq 2 \mu\text{g/ml}$  reduced the total biomass of all mixed biofilms (Fig. 1) and single biofilms of *C. albicans* and *S. aureus* ATCC 29213 and ATCC 6538 strains, only concentrations of  $\geq 32 \mu\text{g/ml}$  CAS inhibited the MRSA strain ATCC 33591 (Fig. S2).

The cell viability of *C. albicans* and *S. aureus* in both mixed and monoculture biofilms was significantly reduced by 128  $\mu\text{g/ml}$  CAS, except for a single biofilm of *S. aureus* ATCC 29213 (Fig. 1, Fig. S2). Interestingly, the cell viability of *C. albicans* in mixed

**TABLE 1** BIC of CAS, MFG, and VCM against mixed biofilms (during their formation and 24 h preformed) of *C. albicans* and *S. aureus*<sup>a</sup>

Biofilm development stage	CAS		MFG		VCM	
	BIC <sub>50</sub>	BIC <sub>90</sub>	BIC <sub>50</sub>	BIC <sub>90</sub>	BIC <sub>50</sub>	BIC <sub>90</sub>
Biofilm formation						
<i>C. albicans</i> SC5314 + <i>S. aureus</i> ATCC 29213	16	128	32	>256	>256	>256
<i>C. albicans</i> SC5314 + <i>S. aureus</i> ATCC 33591	32	64	16	>256	>256	>256
<i>C. albicans</i> SC5314 + <i>S. aureus</i> ATCC 6538	8	32	16	>256	>256	>256
<i>C. albicans</i> IAL-40 + <i>S. aureus</i> ATCC 29213	2	64	≤0.125	0.25	1	16
<i>C. albicans</i> IAL-40 + <i>S. aureus</i> ATCC 33591	8	128	0.5	16	1	>256
<i>C. albicans</i> IAL-40 + <i>S. aureus</i> ATCC 6538	1	32	≤0.125	>256	1	64
24 h preformed biofilm						
<i>C. albicans</i> SC5314 + <i>S. aureus</i> ATCC 29213	128	>256	>256	>256	>256	>256
<i>C. albicans</i> SC5314 + <i>S. aureus</i> ATCC 33591	256	>256	>256	>256	>256	>256
<i>C. albicans</i> SC5314 + <i>S. aureus</i> ATCC 6538	64	>256	>256	>256	>256	>256
<i>C. albicans</i> IAL-40 + <i>S. aureus</i> ATCC 29213	64	>256	2	>256	0.5	>256
<i>C. albicans</i> IAL-40 + <i>S. aureus</i> ATCC 33591	32	>256	32	>256	8	>256
<i>C. albicans</i> IAL-40 + <i>S. aureus</i> ATCC 6538	8	128	16	>256	8	>256

<sup>a</sup>BIC values are micrograms per milliliter and were assigned as a modal average ( $n = 12$ ).

biofilm with *S. aureus* ATCC 6538 had a greater reduction at  $\geq 2 \mu\text{g/ml}$  CAS (ca. 50%), whereas only 128  $\mu\text{g/ml}$  CAS was able to reduce the bacterial viability (Fig. 1F).

#### Caspofungin induces alterations in the cellular morphologies of mixed biofilms.

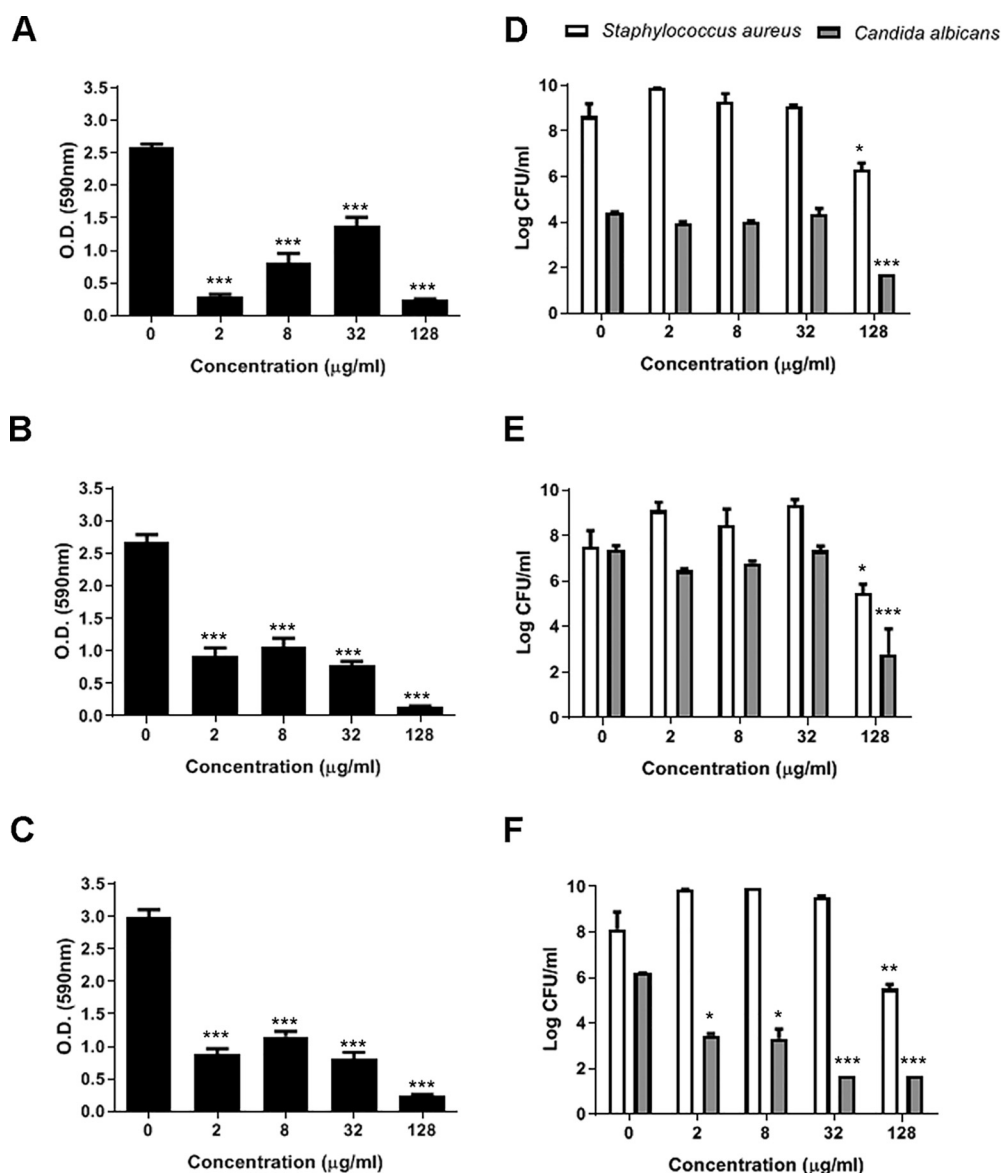
Cell morphology alterations in mixed biofilms, induced by 128  $\mu\text{g/ml}$  CAS, were monitored, since this concentration significantly reduced viable cells of fungi and bacteria. The untreated mixed biofilms showed microbial cell integrity with bacterial cells adhering on yeast/pseudohyphae of *C. albicans* as well as on the catheter surface (Fig. 2A to C), whereas CAS reduced adhesion of microbial cells (Fig. 2D to F). Strikingly, MRSA cells were practically absent from the mixed biofilm after CAS treatment (Fig. 2E). Additionally, CAS induced morphological alterations in both pathogens, where bacterial cell wall was disrupted, and fungal cells showed an enlargement and cell wall disruption (Fig. 2D to F).

**Caspofungin effectiveness against coinfecting *Galleria mellonella* larvae.** Notably, CAS (20 or 50 mg/kg of body weight) treatments increased the health status and survival of larvae coinfecting with *C. albicans* and *S. aureus* (MRSA or MSSA) compared to the untreated group ( $P < 0.0001$ ) (Fig. 3). In addition, an important and significant reduction in the fungal ( $\sim 1$  log) and bacterial (1 to 2 logs) burden (Fig. 4A and D) with induction of granuloma formation was observed in the coinfecting larvae (arrows in Fig. 4), indicating that CAS contributed to containing the polymicrobial infections in *G. mellonella*.

## DISCUSSION

Echinocandins are noncompetitive inhibitors of  $\beta$ -1-3-glucan synthase, an enzyme critical to the synthesis of  $\beta$ -1-3-glucan, which is a major component of the fungal cell wall and ECM from *C. albicans* biofilms. On the other hand, *N*-acetylglucosamine transferase from *S. aureus* is an important enzyme for synthesis of poly- $\beta$ -(1,6)-*N*-acetylglucosamine polysaccharide (PNAG; an ECM component) and the polysaccharide intercellular adhesin (PIA) (12). Due to the homology between *N*-acetylglucosamine transferase and  $\beta$ -1,3-glucan synthase, we have investigated the effect of echinocandins, especially CAS, on mixed biofilm formation and preformed biofilm by *C. albicans* and *S. aureus* (MSSA and MRSA) and toward coinfection using the *Galleria mellonella* invertebrate model.

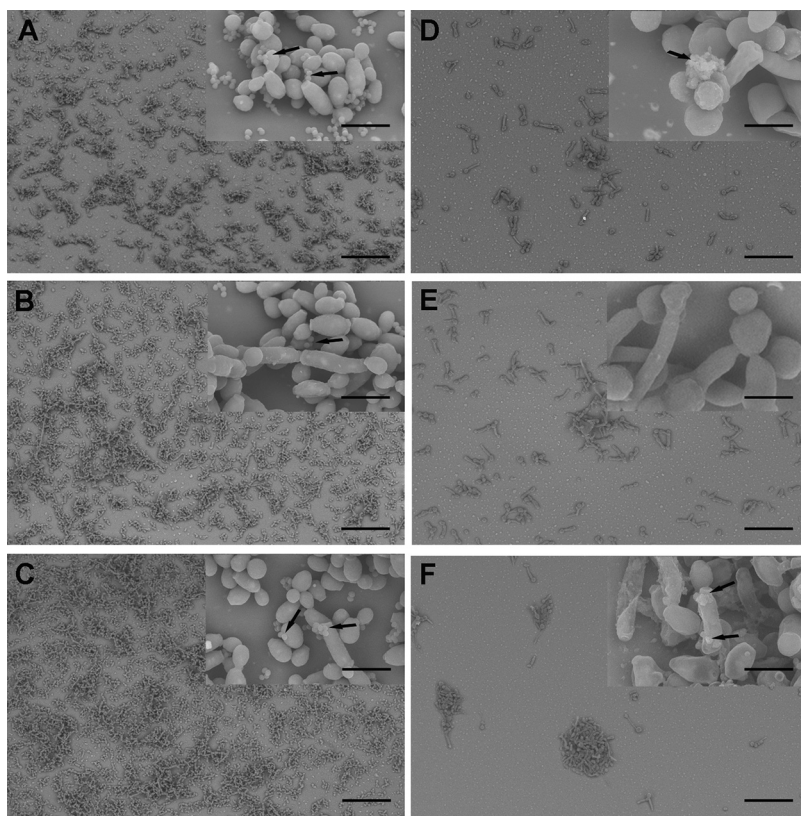
We showed that only CAS showed inhibitory and bactericidal effects on *S. aureus* planktonic cells, confirming previous studies (12). Inhibitory and bactericidal effects of CAS against planktonic cells of VCM-resistant *Enterococcus faecium* have also been demonstrated at 32 and 256  $\mu\text{g/ml}$ , respectively (15). Here, both CAS and MFG inhibited the single- and mixed-biofilm formation by *C. albicans* and *S. aureus*, but CAS was able to inhibit the biofilm formation at lower concentrations; importantly, only CAS inhibited the preformed mixed biofilms. After CAS treatment, a disruption of cell wall



**FIG 1** Total biomass (left) and microbial viability (right) of mixed biofilms of *Candida albicans* and *Staphylococcus aureus* after caspofungin treatment during biofilm formation. (A and D) *C. albicans* SC 5314 and *S. aureus* ATCC 29213. (B and E) *C. albicans* SC 5314 and *S. aureus* ATCC 33591. (C and F) *C. albicans* SC 5314 and *S. aureus* ATCC 6538. The assays were performed in triplicate at least three times. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with the untreated group (ANOVA one-way followed by Dunnett's test).

and enlargement of *C. albicans* were observed as previously described when fungi are treated at high concentrations of CAS (18). Moreover, CAS led to cell wall disruption of *S. aureus* in the mixed biofilms, corroborating alterations also observed on the *E. faecium* cell wall in its planktonic form (15).

The interaction of *C. albicans* and *S. aureus* is considered synergistic, and the quorum sensing molecules are involved in the cellular communication and provide biofilm formation (2). *C. albicans* supplies increased bacterial growth and upregulates the virulence factors and antimicrobial resistance (2, 19–21), while *S. aureus* induces increased *C. albicans* growth and hypha formation (22, 23). Importantly, mixed *C. albicans*-*Staphylococcus* species biofilms display an additional enhanced virulence and tolerance toward antimicrobials compared to their single-species biofilms (2). The interruption of the tridimensional



**FIG 2** Images of scanning electron microscopy of mixed biofilms of *Candida albicans* and *Staphylococcus aureus* left untreated (left) or treated with caspofungin (128  $\mu\text{g/ml}$ ) (right). (A and D) *C. albicans* SC 5314 and *S. aureus* ATCC 29213. (B and E) *C. albicans* SC 5314 and *S. aureus* ATCC 33591. (C and F) *C. albicans* SC 5314 and *S. aureus* ATCC 6538. Bars, 50  $\mu\text{m}$ ; inset bars, 5  $\mu\text{m}$ .

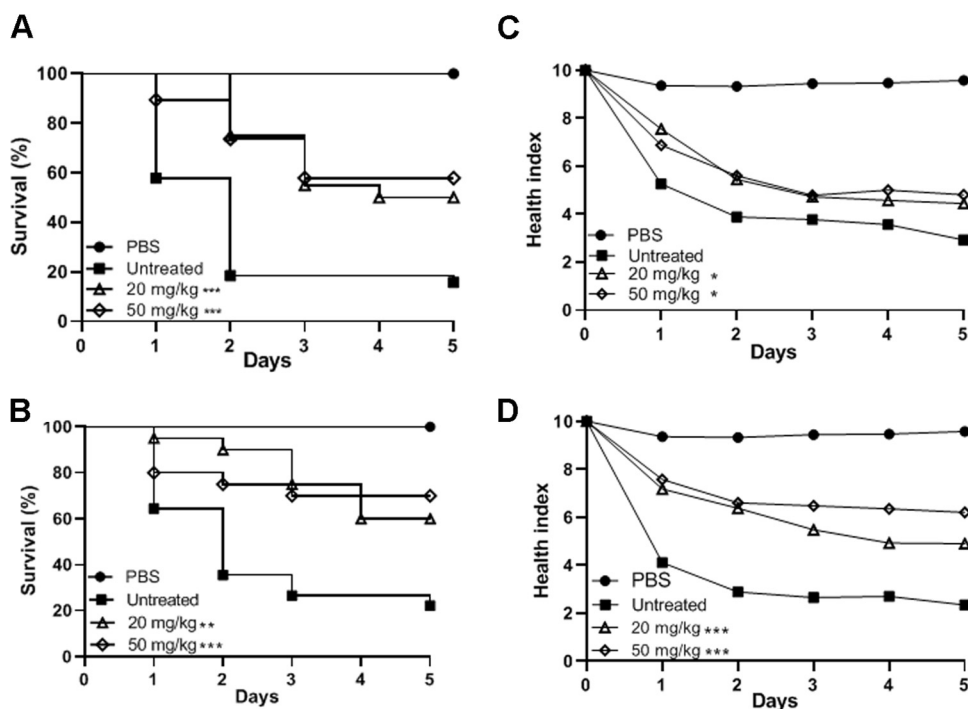
structure formation of biofilms and interkingdom cellular communication then can be considered a relevant strategy for prevention and eradication of mixed biofilms.

The adherence phase is too pivotal for fungus-bacterium interaction and biofilm formation. *S. aureus* cells predominantly adhere to *C. albicans* hyphae, resulting in a unique biofilm architecture (4), and the Als3 protein was identified as a hypha-specific receptor that binds bacteria (24); however, other studies showed that *C. albicans* morphogenesis is not required for their interaction (25). In addition, the interaction can occur between bacteria and yeasts, where cell wall molecules or nonspecific hydrophobic and electrostatic interactions may play a role in interspecies interaction (24, 25). Accordingly, CAS inhibits *C. albicans* yeast-hypha morphogenesis and interrupts the cell wall biosynthesis of both bacterium and fungus, hampering their cell-cell interaction and, consequently, the 3D structure of biofilms as observed here and in previous studies (12, 26).

The ECM, formed by polysaccharides, proteins, and extracellular DNA, assumes an important role in enhanced tolerance to antimicrobials that physically limit penetration of drugs into the biofilms as well as the persister cell differentiation and upregulation of drug efflux pumps (27). In the mixed biofilms, the presence of *C. albicans* appeared to protect *S. aureus* cells from elimination by VCM, an antibiotic normally effective against MRSA, due to the protection of bacterial cells by the ECM produced by *C. albicans* (28). On the other hand, this protective effect is reduced when the production of  $\beta$ -1,3-glucan was interrupted, facilitating the penetration of VCM into biofilms (28).

CAS directly reduces ECM by inhibition of polysaccharide synthesis in *C. albicans* (26) and *S. aureus* (12). In addition, CAS inhibits peptidoglycan synthesis in the Gram-positive bacterium *E. faecalis*, accumulating mucopeptide precursors (15), suggesting





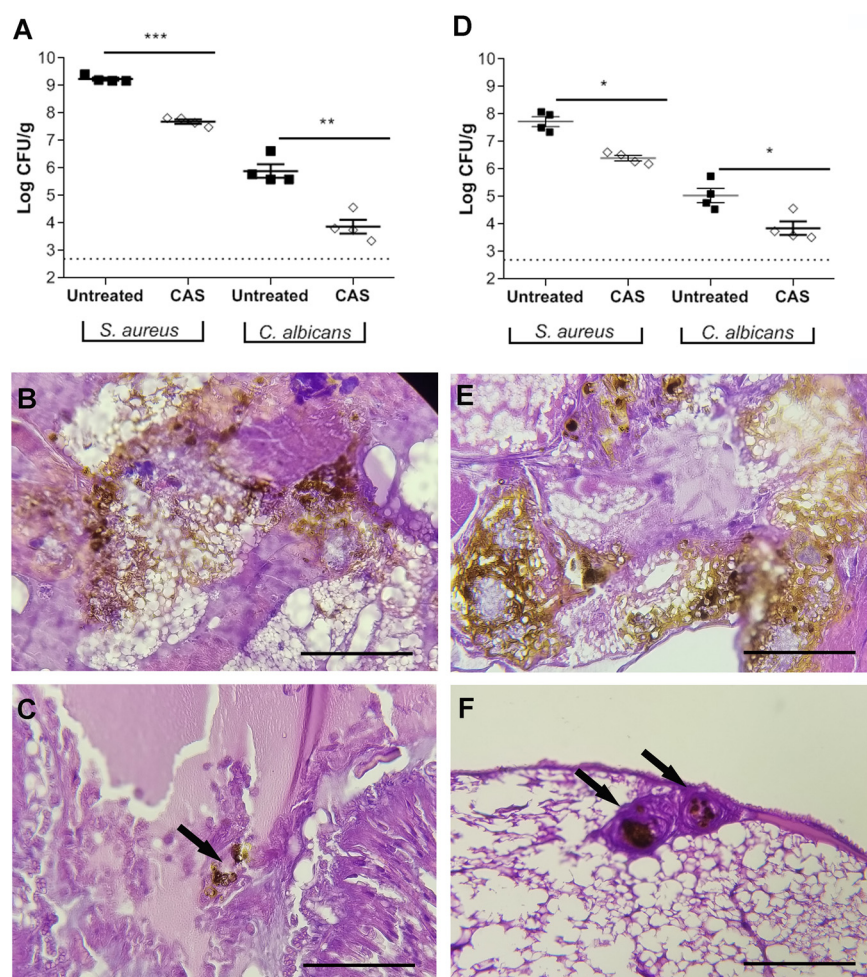
**FIG 3** Survival (left) and morbidity (right) curves of *Galleria mellonella* larvae coinfected with *Candida albicans* and *Staphylococcus aureus* and treated with caspofungin. (A and C) *C. albicans* SC 5314 and *S. aureus* ATCC 33591 (MRSA). (B and D) *C. albicans* SC5314 and *S. aureus* ATCC 6538 (MSSA). \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with the respective untreated group (log-rank [Mantel-Cox] test).

that alteration of cell wall composition could also occur in *S. aureus* cells achieving the cell wall disruption observed here. In this regard, the perturbation of cell wall synthesis in *S. aureus* induces strong repression of the autolytic system by, e.g., subinhibitory concentrations of  $\beta$ -lactam antibiotics (29), impacting the reduction of eDNA important to the ECM composition. The ECM reduction of biofilms then may directly benefit the antibiofilm activity of antimicrobials such as echinocandins, which act as a facilitator agent for enhanced penetration of drug in the deeper layers of biofilms (12, 14).

Therefore, due to the mechanism of action of echinocandins, they can give an important advantage in the treatment of polymicrobial infections in which *Candida* and *Staphylococcus* species are involved. Our results showed the effectiveness of CAS in a coinfection model of *C. albicans* and *S. aureus* in the *G. mellonella* larvae, resulting in increased larval survival and reduced fungal and bacterial burden. In murine models, previous studies showed that CAS combined with fluoroquinolones was effective against *S. aureus* (12), as was anidulafungin combined with tigecycline against *S. aureus*-*C. albicans* coinfection (14). It is important to emphasize that the echinocandins are considered safe and well-tolerated antifungal drugs (30).

A limitation of this study was the absence of a murine model to evaluate the effectiveness of treatments. Instead, we used the *G. mellonella* model. In this regard, new global rules and a modified perception of ethical consciousness have entailed a more rigorous control of utilizations of vertebrates for *in vivo* studies, where numerous alternatives to rodents have been proposed (31). Among these, *G. mellonella* has played a preponderant role, especially in the microbiological field, as demonstrated by the growing number of recent scientific publications. The reasons for its success must be sought in its peculiar characteristics, such as the innate immune response mechanisms and the ability to grow at a temperature of 37°C (31).

In conclusion, CAS showed a potential effect on the mixed biofilms of *C. albicans* and *S. aureus* (MSSA and MRSA strains) in reducing the total biomass as well as the microbial viability. Notably, CAS was able to control the coinfection of *C. albicans* and *S.*



**FIG 4** Microbial burden and histopathological analysis of the larval tissues of *Galleria mellonella* coinfecting with *Candida albicans* and *Staphylococcus aureus* and treated with caspofungin (CAS) at 50 mg/kg. (A to C) *C. albicans* SC 5314 and *S. aureus* ATCC 33591 (MRSA). (D to F) *C. albicans* SC 5314 and *S. aureus* ATCC 6538 (MSSA). (A and D) Microbial burden. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  compared with the untreated group (two-way ANOVA). Dotted lines represent the technique detection limit. Histopathology was for samples untreated (B and E) and treated with CAS (C and F); the black arrows indicate the granulomas, and all images were captured at a magnification of  $\times 400$ . Bars, 25  $\mu\text{m}$ .

*aureus*, increasing the survival and improving the health index of *G. mellonella* larvae and leading to a relevant reduction of microbial burden. Therefore, our results highlight the potential use of CAS in the treatment of polymicrobial infections by *C. albicans* and *S. aureus*; however, further studies should be conducted to refine our findings and improve the therapeutic schemes.

## MATERIALS AND METHODS

**Microorganisms.** *Candida albicans* (SC 5314 and IAL-40) and *Staphylococcus aureus* (ATCC 29213, ATCC 33591, and ATCC 6538) were stored in brain heart infusion and trypticase soy broths, respectively, with 20% glycerol at  $-80^{\circ}\text{C}$ . Yeasts were recovered in Sabouraud dextrose agar and bacteria in trypticase soy agar and subcultured in the same medium at least twice at  $35^{\circ}\text{C}$  for 24 h to obtain optimal microbial growth before assays.

**Antimicrobials.** Caspofungin (CAS), micafungin (MFG), and vancomycin (VCM) (all from Sigma-Aldrich Co., MO, USA) were dissolved in dimethyl sulfoxide to obtain 100-times-concentrated stock solutions and stored at  $-20^{\circ}\text{C}$  for use in the tests.

**Antimicrobial susceptibility testing for planktonic cells.** The antibacterial profile of *S. aureus* strains was determined by diffusion disk test, and the broth microdilution assay was performed to determine the MIC of the antimicrobials against planktonic cells of *S. aureus* and *C. albicans* (32, 33). The

minimum microbicidal concentration was also determined, and it is defined as the lowest concentration that killed 99.9% of microbial cells of the initial inoculum (16).

**Antimicrobial activity on monomicrobial and polymicrobial biofilms.** Here, we tested the anti-biofilm effect of antimicrobials in two phases of biofilm development, during biofilm formation and on preformed biofilms, formed by *C. albicans* (SC5314 and IAL-40) and *S. aureus* (ATCC 29213, ATCC 33591, and ATCC 6538). The microbial inoculum at  $1 \times 10^6$  CFU/ml (bacteria and fungi) was standardized in the RPMI 1640 medium buffered with 0.165 M 3-(*N*-morpholino)propane sulfonic acid (here simply called RPMI). A 100- $\mu$ l aliquot of a single microorganism was dispensed in the 96-well flat-bottomed polystyrene microplate containing 100  $\mu$ l of RPMI for monomicrobial biofilm formation, and 100  $\mu$ l of bacteria and 100  $\mu$ l of fungi were dispensed in the same well for polymicrobial biofilm formation. The microplate then was incubated at 35°C for 1.5 h (adhesion phase) with shaking (150 rpm). Next, the medium was withdrawn, the well washed twice with PBS, and 100  $\mu$ l of RPMI was added to each well to allow biofilm formation by incubation at 35°C with shaking (150 rpm) for 24 h. Wells with untreated cells (drug-free) and medium alone were used as controls for biofilm formation and medium sterility, respectively. To evaluate antimicrobial activity on biofilm formation, 100  $\mu$ l of RPMI containing CAS, MFG, or VCM (0.125 to 256  $\mu$ g/ml) was added to each well after the adhesion phase, and the plates were incubated for 24 h at 35°C, with shaking (150 rpm). To evaluate the effect of antimicrobials on sessile cells of preformed biofilms, the supernatants were removed from each well after 24 h of incubation, and the sessile cells were treated with antimicrobials for 24 h at 35°C, with shaking (150 rpm).

**Violet crystal staining assay.** After antimicrobial treatments, the total biomass of bacterial, fungal, and mixed biofilms was quantified using violet crystal staining (16). The optical density (O.D.) was determined, and the inhibition percentage of antimicrobials was calculated by following the formula  $100 - [(treated\ cells\ O.D. \times 100)/untreated\ cells\ O.D.]$  for determination of the lowest concentrations that inhibit 50% and 90% of biofilm formation ( $BIC_{50}$  and  $BIC_{90}$ , respectively) (16).

**Fungal and bacterial viability.** To evaluate the cell viability in the mono- and polymicrobial biofilms, a CFU counting assay was performed (16). After the adhesion phase, the cells were treated with CAS at 2, 8, 32, or 128  $\mu$ g/ml in RPMI medium for 24 h at 35°C, with shaking (150 rpm). Next, the sessile cells were washed twice in PBS and removed by scrapping for CFU counts using Sabouraud dextrose agar containing 50  $\mu$ g/ml chloramphenicol for *C. albicans* and mannitol salt agar for *S. aureus* to further calculate of log CFU/ml values (16).

**Scanning electron microscopy.** Mixed biofilms of *C. albicans* and *S. aureus* were treated with 128  $\mu$ g/ml CAS after the adhesion phase on the surface of a catheter section of 5 mm for 24 h at 35°C, with shaking (150 rpm). The biofilms were washed twice with PBS and fixed using 2.5% glutaraldehyde in PBS for 1 h at room temperature. The biofilms then were dehydrated in increasing concentrations of ethanol, dried using hexamethyldisilazane (HMDS; Sigma-Merck), and coated with platinum for observation in a scanning electron microscope (Quanta 650 FEG; FEI, Thermo Scientific, Hillsboro, OR, USA).

**Antimicrobial efficacy of caspofungin against coinfection of *C. albicans* and *S. aureus* using the *Galleria mellonella* model.** *C. albicans* and *S. aureus* (MSSA or MRSA) were used for mixed infection in the *G. mellonella* larvae (ca. 200 mg of body weight) that were obtained in the laboratory at controlled temperature (30°C) using beeswax and pollen as food. For systemic infection, a volume of 10  $\mu$ l of mixed microbial suspension ( $5 \times 10^5$  CFU for yeast and  $1 \times 10^7$  CFU for bacteria) in PBS was inoculated in the last larval proleg with a Hamilton syringe. After 30 min of infection, CAS (20 or 50 mg/kg) was administered systemically in another larval proleg for treatments of mixed infections. Infected and untreated larvae (untreated group) and uninfected larvae (PBS group) received only PBS and were included in the assay as control groups for microbial infection and mechanical trauma by injections. A total of 20 larvae were used for each group and incubated at 37°C. The larval survival and health status were monitored every 24 h for up to 5 days after treatments for construction of the survival and morbidity curves, respectively (34). The microbial burden was determined 24 h postinfection ( $n = 4$  larvae/group) by CFU counting assay using Sabouraud dextrose agar containing chloramphenicol (50  $\mu$ g/ml) for *C. albicans* and salt mannitol agar for *S. aureus* to obtain log CFU/g values. The histological analysis was performed using 2 larvae from each group fixed with 4% formaldehyde in PBS and prepared for the histological sections and staining with hematoxylin and eosin (HE) (35).

**Statistical analysis.** Statistical analyses were performed using the software Prism version 8.0 (GraphPad, La Jolla, CA), and *P* values of  $<0.05$  were considered significant.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

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G.S. and B.N.F. performed the experiments, analyzed the results, and drafted the manuscript. K.I. designed the experiments, analyzed the data, and wrote and edited the manuscript. N.L. did a critical analysis and edited the manuscript. All authors have read and approved the manuscript before publication.

We declare no conflicts of interest.

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