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Susceptibility of *Candida glabrata* biofilms to echinocandins: alterations in the matrix composition

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ABSTRACT

Candidiases are the most recurrent fungal infections, especially among immunosuppressed patients. Although *Candida albicans* is still the most widespread isolated species, non-*Candida albicans Candida* species have been increasing. The goal of this work was to determine the susceptibility of *C. glabrata* biofilms to echinocandins and to evaluate their effect on the biofilm matrix composition, comparing the results with other *Candida* species. Drug susceptibilities were assessed through the determination of minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and minimum biofilm eradication concentration (MBEC) of caspofungin (Csf) and micafugin (Mcf). The β-1,3 glucans content of the matrices was assessed after contact with the drugs. The data suggest that, generally, after contact with echinocandins, the concentration of β-1,3 glucans increased. These adjustments in the matrix composition of *C. glabrata* biofilms and the chemical differences between Csf and Mcf, seem responsible and may determine the effectivity of the drug responses.

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Introduction

Infections caused by members of the genus *Candida* (candidiasis) have been increasing in recent decades and becoming more difficult to eradicate. There are about 15 different *Candida* species that cause infections in humans, and *Candida glabrata* is one of the most common (Pappas 2006; Pappas et al. 2015). Candidiasis generally occurs due to the unbalanced use of immunosuppressive drugs, broad spectrum antibiotics and the widespread use of indwelling medical devices, but also to the growth of immunogenic diseases, the upsurge of endocrine disorders, and the ageing and the increase in the patient population (Douglas 2003; Li et al. 2007; Garcia-Cuesta et al. 2014; Silva et al. 2017). Each *Candida* species has distinctive virulence factors, antifungal susceptibilities, and defined epidemiologies (Pappas et al. 2015). The aptitude of these organisms to form biofilms is a specific virulence feature which allows tissue attachment following infection of the host (McCall and Edgerton 2017). Biofilms are communities of microorganisms embedded in an extracellular matrix (Costerton et al. 1995; Donlan and Costerton 2002), which confers significant resistance to antifungal therapy and intense host immune responses (Fonseca et al. 2014; Rodrigues et al. 2014). This matrix is composed of exopolymeric compounds secreted by sessile cells, with all providing protection against environmental challenges (Pierce et al. 2017). Infections caused by biofilms are complicated due to inducible gene networks encoding different proteins that confer tolerance or resistance to many of the available antifungal drugs (Ramage et al. 2012).

Chemically, echinocandins are cyclic lipo-hexapeptides with modified N-linked acyl lipid side chains (Chang et al. 2017), biosynthesised by diverse members of the Ascomycota (fungi) on non-ribosomal peptide synthase complexes (Emri et al. 2013). The first echinocandin with antimycotic activity was discovered in the 1970s, and since, over 20 natural echinocandins have been isolated (Emri et al. 2013). By disturbing fungal cell wall synthesis through a non-competitive inhibition of β-1,3-glucan synthesis, these drugs weaken the cell wall, break down the cellular integrity and, finally, induce cell lysis (Debono and Gordee 1994; Perlin et al. 2007). Due to this mechanism of action the echinocandins (which include anidulafungin, caspofungin and micafungin) are generally well tolerated, avoiding the overlapping toxicities and drug–drug interactions with mammalian cells which are observed with the azoles and the polyenes (Wiederhold and Lewis 2003; Wiederhold et al. 2007; Chang et al. 2017). The high clinical efficacy in the non-neutropenic

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patient population, in patients with moderate to severe illness, and in patients with pre-azole exposure (Pappas et al. 2015; Perlin 2015b), resulted in echinocandins being recommended as the first-line antifungal agents to treat invasive candidiasis, especially *Candida glabrata*, due to its innate high azole resistance (Perlin 2007; Pappas et al. 2015). Although these antifungal drugs are active against most important *Candida* species, in which they display *in vitro* fungicidal activity (Barchiesi et al. 2005), in critically ill patients it is recognised that the achievement of their pharmacodynamic and pharmacokinetic targets show a large inter-individual variability (Chang et al. 2017). In Europe, micafungin (Mcf) is approved for use in paediatric patients of any age including neonates, while caspofungin (Csf) is approved for use in paediatric patients ≥ 1 year old, since there are insufficient data regarding its use in those <1 year old (Tragiannidis et al. 2012; Viscoli et al. 2014). The use of Csf and Mcf is limited by the necessity for a once-daily intravenous dosage regimen, lack of an oral formulation and a limited spectrum (Barchiesi et al. 2005; Pappas et al. 2015; McCarty and Pappas 2016), but both echinocandins still show very good *in vitro* activity against clinically relevant isolates of *Candida* species (Pappas et al. 2007; Spreghini et al. 2012; Kohno et al. 2013; Perlin 2015a; Chapman et al. 2017). In trials involving adult and paediatric patients with invasive and oesophageal candidiasis, Mcf has been shown to be non-inferior to intravenous Csf, intravenous fluconazole or liposomal amphotericin B. The tolerability profile of Csf and Mcf are, in general, similar to fluconazole and are better tolerated than liposomal amphotericin B or oral itraconazole (Scott 2012).

The biofilm matrices of *Candida* species have a strong net of exopolymers, providing protection against physical and chemical environmental attack, such as by drugs. These polymers make it difficult for drugs to diffuse into the biofilm cells, which make the biofilms recalcitrant to antifungals (Al-Fattani and Douglas 2004; Rodrigues, Gonçalves et al. 2017; Dominguez et al. 2018). Hence, the goal of this work was to evaluate seven *C. glabrata* isolates, by comparison with *C. albicans*, *C. parapsilosis* and *C. tropicalis*, regarding their susceptibility in the planktonic and biofilm form to Csf and Mcf, and the biochemical variations induced in the composition of the matrices after the drug exposure, in order to relate these matrix variations with drug effectiveness.

Material and methods

Organisms

A total of 10 strains were used in the course of this study. Six clinical isolates of *C. glabrata* from Hospital Escala Braga in Portugal were recovered from different sites: oral cavity (*C. glabrata* AE2 and D1), urinary tract (*C. glabrata* 562123 and 513100), vaginal tract (*C. glabrata* 534784 and 585626); four reference strains were from the American Type Culture Collection (*C. glabrata* ATCC2001, *C. albicans* SC5314 (ATCCMYA2876), *C. parapsilosis* ATCC22019 and *C. tropicalis* ATCC750). The identity of all isolates was confirmed using CHROMagar *Candida* (CHROMagar, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8s subunit gene reference. Genomic DNA was extracted following previously described procedures (Williams et al. 1995). The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck) and incubated for 18 h at 37°C, under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation, 3,000 *g* for 10 min, at 4°C and washed twice with phosphate buffer saline (PBS, 0.1 *M*, pH=7.5; NaCl 0.8%, KCl 0.02%, K_2HPO_4 0.02%, $NaHPO_4.12H_2O$ 0.285%). Pellets were then suspended in RPMI-1640 (pH=7, Sigma-Aldrich, St Louis, MO, USA) and the cellular density was adjusted to $1{\times}10^5$ cells ml^{-1}, using a Neubauer counting chamber.

Antifungal drugs

Csf and Mcf were kindly provided by MSD® and Astellas®, respectively. Aliquots of 5,000 mg l^{-1} were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with RPMI-1640 (Sigma-Aldrich) for both drugs.

Antifungal susceptibility tests

All the antifungal susceptibility tests were performed using the microdilution method, in accordance to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST [date unknown]; Arendrup et al. 2008).

Minimum inhibitory concentrations (MICs)

The inoculum was prepared by suspending five distinct colonies, ≥1 mm diameter from 24 h cultures, in at least 3 ml of sterile distilled water. Then, the inoculum was suspended by vigorous shaking on a vortex mixer for 15 s and the cell density was adjusted to the density of a 0.5 McFarland standard and adding sterile distilled water as

required, giving a yeast suspension of $1–5 \times 10^6$ CFU ml^{-1} (colony forming units). A working suspension was prepared by a dilution of the standardised suspension in sterile distilled water to yield $1–5 \times 10^5$ CFU ml^{-1}. The 96-well plate was prepared with 100 µl of cell suspension and 100 µl of each antifungal agent (Csf: 0.001 to 0.075 mg l^{-1}; Mcf: 0.002 to 0.2 mg l^{-1} – 2x concentrated) and incubated at 37°C, during 18–48 h. Controls without antifungal agents were also performed (positive control: working solution of cells and RPMI-1640; negative control: sterile distilled water and RPMI-1640). Finally, the results were determined spectrophotometrically at 530 nm and the MIC was considered to be the value that inhibited 50%, compared to the controls (according to EUCAST guidelines).

Minimum fungicidal concentration (MFC)

In addition to the previous step, 20 µl of each cell suspension treated with Csf and Mcf were recovered to a new well and serial decimal dilutions in PBS were plated onto SDA. Agar plates were incubated for 24 h at 37°C, and the total number of CFUs was determined. The results were presented as Log_{10} CFU per unit area (Log_{10} CFU cm^{-2}) (Rodrigues and Henriques 2017).

Minimum biofilm eradication concentration (MBEC)

Standardised cell suspensions (200 µl) were placed into selected wells of 96-well polystyrene microtitre plates (Orange Scientific, Braine-l'Alleud, Belgium). RPMI-1640 was used without cells, but without antifungal agent, as a negative control. As positive control, cell suspensions were tested with RPMI-1640, without the antifungal agent. At 24 h, 100 µl of RPMI-1640 were removed and an equal volume of fresh RPMI-1640 plus the respective antifungal concentration was added (Csf: 0.5 to 3 mg l^{-1}; Mcf: 3 to 17 mg l^{-1}; 2× concentrated). The plates were incubated at 37°C for more 24 h, a total of 48 h, at 120 rpm. The number of cultivable cells on biofilms was determined by the enumeration of CFUs. For that, after the period of biofilm formation, all medium was aspired and the biofilms washed once with 200 µl of PBS to remove non-adherent cells. Then, biofilms were scraped from the wells and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Serial decimal dilutions in PBS were plated on SDA and incubated for 24 h at 37°C. The results were presented as total CFUs per unit area (Log_{10} CFU cm^{-2}) (Rodrigues, Gonçalves et al. 2017).

Biofilm analysis

Biofilm total biomass quantification – crystal violet staining

Total biofilm biomass was quantified by crystal violet (CV) staining in 96-well plates (Rodrigues and Henriques 2017). As described previously, after biofilm formation for a total of 48 h, the medium was aspirated and non-adherent cells removed by washing the biofilms with PBS. Then, biofilms were fixed with 200 µl of methanol, which was removed after 15 min of contact. The microtitre plates were allowed to dry at room temperature, and 200 µl of CV (1% v v^{-1}) were added to each well and incubated for 5 min. The wells were then gently washed twice with sterile, ultra-pure water and 200 µl of acetic acid (33% v v^{-1}) were added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtitre plate reader (Bio-Tek Synergy HT, Izasa, Lisbon, Portugal) at 570 nm. The results were presented as absorbance per unit area (Abs cm^{-2}).

Biofilm structure visualisation

In order to examine the structure of biofilms, after biofilm formation in the presence or absence of both drugs, they were observed by scanning electron microscopy (SEM). For that, biofilms formed as described above were dehydrated with ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air dried for 20 min. Samples were kept in a desiccator until further analysis. Prior to observation, the base of the wells was mounted onto aluminium stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, MA, USA) (Rodrigues and Henriques 2017).

Biofilm matrix composition evaluation

Extraction method: biofilms were formed in 24-well polystyrene microtitre plates (Orange Scientific) (Rodrigues, Gonçalves et al. 2017). For this, 1,000 µl of yeast cell suspension (1×10^5 cells ml^{-1} in RPMI-1640 or RPMI-1640 with Csf and Mcf at concentrations corresponding to the MBEC of each species/strain) were added to each well and the biofilms were treated as described previously (Rodrigues, Gonçalves et al. 2017). After 24 h, 500 µl of RPMI-1640 medium were removed and an equal volume of fresh RPMI-1640 with or without the antifungal agents was added. After another 24 h, the biofilms were scraped from the 24-well plates, resuspended in ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Vernon Hills, IL, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min. The suspension was centrifuged at 5,000 *g* for 5 min at 4°C. The supernatant (matrix) was filtered through a 0.2 µm nitrocellulose filter and dried at 37°C until a constant dry biofilm weight was reached. The pellets (sessile yeast cells) were discarded.

β-1,3 glucan concentration determination: β-1,3 glucan concentrations were determined using Glucatell® kit (Cape Cod®, East Falmouth, MA, USA). The values were normalised per pg μg^{-1} of β-1,3 glucans/total polysaccharide

content (evaluated by the phenol-sulfuric procedure using glucose as standard (DuBois et al. 1956).

Statistical analysis

All experiments were repeated three times in at least three independent assays. The results were compared using one-way and two-way ANOVA, Tukey's and Dunnett's multiple comparisons test, using GraphPad® Prism® 7 software (San Diego, CA, USA). All tests were performed with a confidence level of 95%.

Results

Planktonic and biofilm susceptibility to Csf and Mcf

Table 1 shows the MIC, MFC and MBEC determined for all the strains used in this study. The values were species/strain dependent, but, in general, MFC and MBEC of Mcf were higher than those from Csf. This was especially noticeable for the concentration needed to eradicate the biofilm (MBEC), which was sometimes 5–6 times higher than the MFC. Mostly, all *C. glabrata* strains were demonstrated to have biofilm resistance profiles similar to the other *Candida* species.

Biofilm reduction capacity of Csf and Mcf

CV staining was used to evaluate the biomass reduction in the *Candida* biofilms, after 24 h contact with Csf or Mcf (Table 2). Although both agents showed a good capacity in reducing the biomass, Csf had a higher capacity than Mcf. Four of the seven *C. glabrata* strains (585626, 534784, D1 and ATCC2001) had the lowest percentage biomass reduction for Csf and Mcf, indicating a more tolerant profile of this species, comparing to the other species of *Candida*. On the other hand, *C. albicans* SC5314 ($p < 0.001$ for Csf and Mcf) was demonstrated to have the most marked biomass reductions.

Biofilm structure

SEM images (Figure 1A and B) confirmed that all *Candida* strains had a good capacity for biofilm production, especially *C. glabrata* AE2, *C. glabrata* D1 (biofilms formed in a long continuous carpet (Hawser and Douglas 1995; Fonseca et al. 2014; Rodrigues and Henriques 2017), *C. albicans* SC5314 (biofilm presenting high hyphal quantity and entanglement (Uppuluri et al. 2010; Rodrigues and Henriques 2017) and *C. parapsilosis* ATCC22019 (continuous biofilm carpet with clumped blastospores (Taff et al. 2012; Rodrigues and Henriques 2017). *C. tropicalis* ATCC750 biofilm can be described as chains of cells with large amounts of extracellular material (Heffner and Franklin 1978; Rodrigues and Henriques 2017; Silva et al. 2017). After Csf and Mcf contact, the SEM images confirmed the CV results for all species/strains (Figure 1B). A biofilm reduction was observed in the presence of both drugs, but especially with Csf, and the biofilm cells presented a concave aspect and appear to have a reduction in the extracellular matrix. However, the extracellular matrix

Table 1. MIC, MFC and MBEC values (mg l^{-1}) for Csf and Mcf of *C. glabrata*, *C. albicans*, *C. parapsilosis* and *C. tropicalis* strains.

Origin	Species/strain		Determination	Caspofungin	Micafungin
Reference	*C. glabrata*	ATCC2001	MIC	0.064	0.032
			MFC	0.10	0.20
			MBEC	**2.5–3**	**16–17**
Oral cavity		AE2	MIC	≤0.003	0.002
			MFC	0.003	0.06
			MBEC	**0.5–1**	**7–8**
		D1	MIC	0.003	0.002
			MFC	0.003	0.06
			MBEC	**2.5–3**	**3–3.5**
Urinary tract		562123	MIC	0.001	0.01
			MFC	0.001	0.10
			MBEC	**0.5–1**	**16–17**
		513100	MIC	0.008	0.01
			MFC	0.008	0.06
			MBEC	**2–2.5**	**16**
Vaginal tract		534784	MIC	≤0.001	0.002
			MFC	0.05	0.06
			MBEC	**2.5–3**	**5.5–6**
		585626	MIC	0.003	0.01
			MFC	0.003	0.06
			MBEC	**2.5**	**5–5.5**
Reference	*C. albicans* SC5314		MIC	0.016	≤0.016
			MFC	0.016	0.06
			MBEC	**2.5–3**	**3.5**
Reference	*C. parapsilosis* ATCC22019		MIC	0.016	>0.032
			MFC	0.08	0.2
			MBEC	**2.5**	**16**
Reference	*C. tropicalis* ATCC750		MIC	0.008	0.002
			MFC	0.016	0.06
			MBEC	**2.5**	**16**

Note: Bold indicates MBEC values.

Table 2. Percentage biomass reduction of *C. glabrata*, *C. albicans*, *C. parapsilosis* and *C. tropicalis* strains after caspofungin and micafungin contact.

Species/strain		% Biomass reduction (*p*-value)	
		Caspofungin	Micafungin
C. glabrata	ATCC2001	82.96 (**)	19.1 (*)
	AE2	79.71 (***)	39.28 (**)
	D1	69.71 (*)	23.84 (ns)
	562123	85.16 (**)	70.47 (**)
	513100	88.47 (****)	66.59 (****)
	534784	73.38 (*)	24.46 (ns)
	585626	77.85 (**)	53.63 (**)
C. albicans SC5314		92.33 (***)	82.13 (***)
C. parapsilosis ATCC22019		78.55 (***)	38.86 (**)
C. tropicalis ATCC750		84.20 (**)	53.49 (*)

Notes: ns, non-significant.
The concentrations applied in each species/strain were those determined by the MBECs. Overall ANOVA $p<0.05$ and *post hoc* Dunnett's comparison test: $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0005$; $^{****}p < 0.0001$

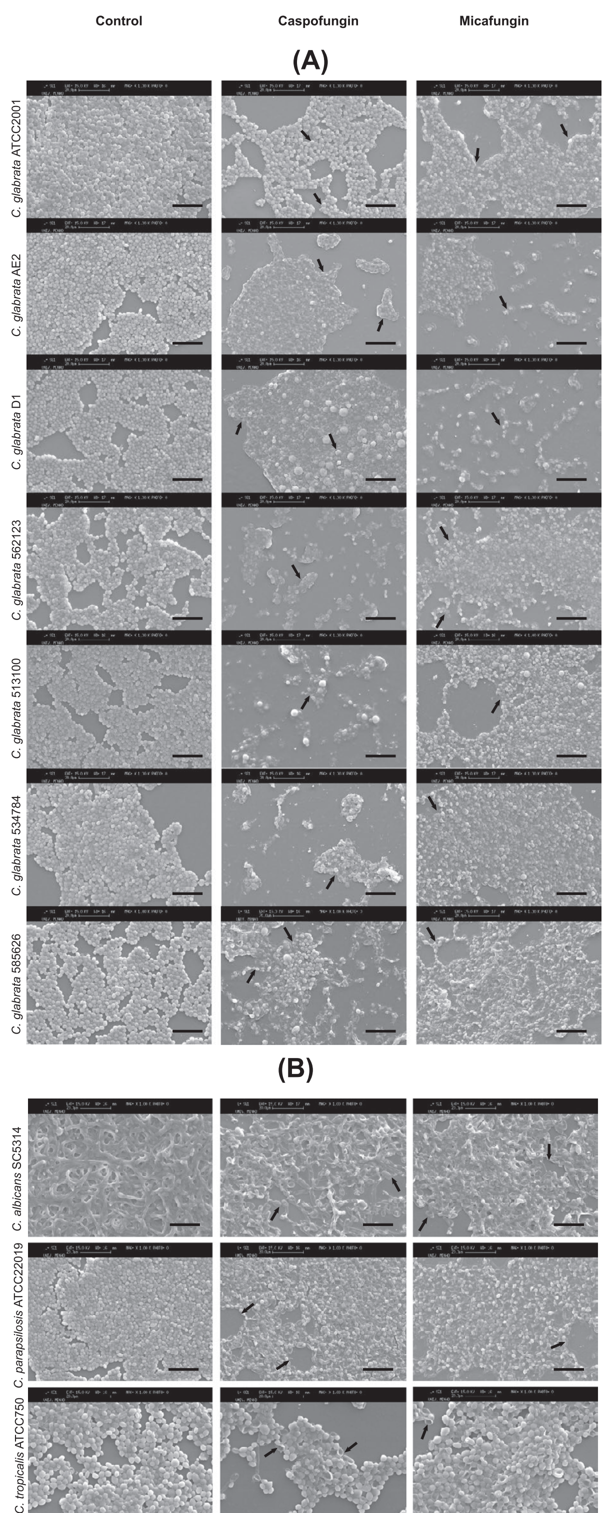

Figure 1. SEM observations of biofilms *C. glabrata* (A), *C. albicans*, *C. parapsilosis* and *C. tropicalis* (B) strains/species grown without drugs (control) and after caspofungin and micafungin contact. The concentrations applied to each species/strain were those determined by the MBECs. The arrows show disrupted biofilms and damaged *Candida* cells. Magnification: 1,000×. Scale bar=20 µm.

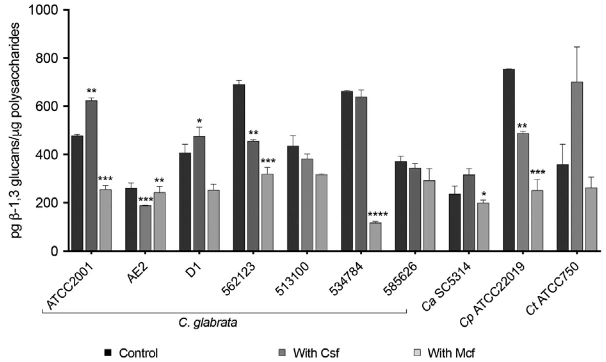

Figure 2. β-1,3 glucans concentration/polysaccharides content (pg μg^{-1}) in 48-h biofilm matrices of *C. glabrata*, *C. albicans* (Ca), *C. parapsilosis* (Cp) and *C. tropicalis* (Ct). Overall ANOVA $p<0.05$ ANOVA and *post hoc* Tukey's comparisons test: $^{*}p<0.05$; $^{**}p<0.001$; $^{***}p<0.0005$; $^{****}p<0.0001$).

of the different biofilms in the presence of the drugs seems to be different, which may be explained by variations in β-1,3 glucans, as was evaluated subsequently.

Matrix composition after Csf and Mcf contact

The results from the determination of the β-1,3 glucans concentration showed that, compared to the control groups and with some exceptions, the β-1,3 glucans generally tend to statistically decrease in the biofilm matrices of all *Candida* species, after contact with Csf and especially with Mcf (Figure 2). This reduction was not so prominent in *C. glabrata*, when compared to the other species (Figure 2).

Discussion

Systemic candidiasis is a growing problem in the hospitals worldwide (Cataldi et al. 2016; Rodrigues, Rodrigues et al. 2017), with high morbidity and mortality rates and elevated economic costs (Pfaller and Castanheira 2016; Silva et al. 2017). The present study evaluated the efficacy of two of the most used echinocandins – the first-line antifungal agents to treat systemic candidiasis (Pappas et al. 2015; McCarty and Pappas 2016) – of seven *C. glabrata* strains compared with the reference strains *C. albicans*, *C. parapsilosis* and *C. tropicalis*.

Considering MIC values of Mcf, EUCAST guidelines indicate breakpoints of 0.032 mg l^{-1} for *C. glabrata*, 0.016 mg l^{1} for *C. albicans*, 0.002–2 mg l^{-1} for *C. parapsilosis*. For *C. tropicalis*, the values are defined as 1–2 twofold dilution steps higher than for *C. albicans* and *C. glabrata*, but EUCAST attest that there is insufficient evidence to

indicate whether the wild-type population can be considered susceptible to Mcf (EUCAST). For MIC Csf values, EUCAST breakpoints have not yet been established, due to significant inter-laboratory variation in the ranges for this drug (EUCAST). Possibly because of this, the authors had difficulty in defining these parameters. The occurrence of paradoxical growth of the isolates of *Candida* species, also enhanced the difficulties in MIC determination. The paradoxical effect is recognised as a robust mechanism of antifungal resistance. It is a resurgence of growth at drug concentrations above the MIC and connected to an increase in chitin biosynthesis whereby susceptible cells show growth at very high drug levels. *Candida* isolates display paradoxical effect more frequently when grown as biofilms compared to the planktonic form (Gow et al. 2007; Gil-Alonso et al. 2015, 2016; Scorzoni et al. 2017). The results, however, confirmed that all species/strains were Mcf susceptible (Table 1). EUCAST guidelines state that isolates that are susceptible to Mcf should be considered susceptible to Csf (EUCAST), hence all species/strains were considered susceptible to this drug (Table 1). Although Mcf concentrations were, in most cases, higher than Csf concentrations, in general, the MIC, MFC and MBEC values were species/strain dependent. *C. glabrata* strains were revealed to have similar MIC, MFC and MBEC profiles to *C. albicans* SC5314, *C. tropicalis* ATCC750 and *C. parapsilosis* ATCC22019. Not surprisingly, the MBEC results evidenced the higher resistant profile of biofilm cells to antifungal agents than planktonic cells (Lewis et al. 2002; Al-Fattani and Douglas 2004; Ferrari et al. 2011; Grandesso et al. 2012; De Luca et al. 2012). In general, *C. glabrata* MIC values were shown to be closer, but slightly lower than those reported in the literature for some strains (Scott 2012; Naicker et al. 2016) and that all the strains were more susceptible to both echinocandins than *C. glabrata* ATCC2001 (EUCAST). Yet, and as supported by the reports, *C. parapsilosis* showed higher MIC values than the other *Candida* species (Arendrup et al. 2012; Arendrup and Pfaller 2012; Arendrup and Perlin 2014; Pham et al. 2014; Gil-Alonso et al. 2015). It is known that some *Candida* species have naturally occurring polymorphisms in their *FKS* genes, which strongly reduce their susceptibility to echinocandin drugs (Arendrup et al. 2012; Perlin 2015a). Moreover, the *C. parapsilosis* family (*C. orthopsilosis* and *C. metapsilosis*) and *Candida guilliermondii*, normally also have higher MIC values compared to other susceptible *Candida* species (Pfaller et al. 2008, 2010; Tortorano et al. 2013; Perlin 2015b).

The results of CV staining indicated, altogether, an effective reduction in the biomass of the species/strains after echinocandin exposure (Table 2). Yet, Csf showed a higher biomass reduction capacity than Mcf, with a minimal reduction of 70% of the biofilms. Similar results have been indicated previously in studies with catheters (Falcone et al. 2009; Dutronc et al. 2010; Williams and Ramage 2015). Mcf did not reveal a complete eradication of the biofilm, as it has been reported for Csf (Cateau et al. 2008, 2011). This conclusion was particularly noticed in *C. glabrata* (four of the seven strains had the lowest Mcf biomass percentage reduction), which can be related to the usual antifungal resistance profile of this species (Jabra-rizk et al. 2004; Farmakiotis et al. 2014; Perlin et al. 2015; Silva et al. 2017). Interestingly, the efficacy of the biomass reduction by Csf was not always related to the use of the highest drug concentration. In some cases, a high Csf concentration would lead to a low drug activity and, thus, to a lower biomass reduction, revealing a probable paradoxical effect (Wiederhold 2009; Perlin 2014; Gil-Alonso et al. 2015; Scorzoni et al. 2017). By applying an adequate concentration for the strain (sometimes a lower drug dose), this effect was not detected, and the biomass was effectively reduced.

In order to evaluate the extent of the antifungal effect of Csf and Mcf on the biofilms, SEM images were taken (Figure 1A and B). The controls revealed that all *Candida* species had a good capacity for biofilm production. After Csf and Mcf contact (Figure 1B), and particularly with Csf, a strong biofilm reduction, was observed (arrows in the Figure 1). The biofilm cells seemed to be disrupted and presented a concave aspect (arrows in Figure 1), endorsing the CV results (Table 2). This disruption and change in the cell configuration is expected to result from the mechanism of action of the echinocandins (non-competitive inhibition of β-1,3-glucan synthesis) (Rodrigues, Rodrigues et al. 2017; Scorzoni et al. 2017), which affects the cell wall and the matrix composition. Fungal cell wall polysaccharides are significant constituents of the biofilm matrix of *Candida* species (Chandra et al. 2001; Kuhn et al. 2002). The biofilm matrix composition of *C. glabrata*, *C. albicans*, *C. parapsilosis* and *C. tropicalis* strains with and without Csf and Mcf contact were evaluated in terms of β-1,3 glucan content (Figure 2). To the authors' knowledge, this is the first report describing such content of the biofilm matrices of *Candida* species in contact with these two echinocandins. The β-1,3 glucans are a group of specific polysaccharides from the cell walls of *Candida* species that are also recognised as major constituents of the biofilm matrices of this genus (Chandra et al. 2001; Kuhn et al. 2002). Regarding the controls (non-treated biofilms), *C. albicans* SC5314 was the species that had a lower quantify of β-1,3 glucans in the matrix and *C. parapsilosis* ATCC22019, *C. glabrata* 534784 and *C. glabrata* 562123 were revealed to have the highest content of this glucan, which could be one of the probable mechanisms for the resistant profile of the biofilms (Figure 2). After adding Csf and Mcf to the pre-formed biofilms, the results

from the determination of the β-1,3 glucan concentrations (Figure 2) demonstrated that these compounds are, in general, statistically significantly likely to reduce in the biofilm matrices of C. *glabrata*, and in the other *Candida* species. Exceptions were noted only after the addition of Csf to *C. glabrata* ATCC2001, *C. glabrata* D1 and *C. tropicalis* ATCC750. It is also important to note that the initial concentration of β-1,3 glucans was very dependent on the strain and that, excepting *C. glabrata* 534784, the variations between the strains and the Mcf groups were less pronounced than those detected between the strains and Csf groups.

Another source of variability in the drug response might be the chemical differences between the two echinocandin molecules. Both Csf and Mcf have a cyclic peptide structure with a N-aryl group but with different patterns of hydroxylations and amino groups (R2 to R4). The N-aryl side chain (position R1) plays a critical role in the potency and toxicity and is the main point for chemical modification of the echinocandin analogues (Debono and Gordee 1994; Wiederhold and Lewis 2003). Csf and Mcf have also variations in R2, R3 and R4 positions: Csf is more hydroxylated and has more amino groups, while Mcf has more aryl groups. It is documented that the replacement of the linoleoyl side chain with aryl side chains of low lipophilicity, nonlinear configuration or chains switched with highly polar groups end in loss of antifungal activity, which suggests that planar, non-polar substitutions are critical for the antifungal activity (Debono and Gordee 1994; Wiederhold and Lewis 2003). These modifications were performed on the Mcf molecule and are also a probable explanation for the differences found, in this work, for the drug activity in the species and strains studied (higher MBEC values and matrix variations, when compared to Csf).

This report shows a general parallelism in the efficacy of the Csf and Mcf susceptibilities of *C. glabrata* in comparison to *C. albicans*, *C. parapsilosis* and *C. tropicalis* with respect to planktonic cells (MIC/MFC). It seems plausible to say that the external alterations in the matrix composition of the *Candida* strains and the chemical alterations in the Csf and Mcf molecules can partially explain and determine the effectiveness response to these two drugs of biofilm infections of *Candida* species and, particularly, *C. glabrata*.

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