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The Effectiveness of Voriconazole in Therapy of *Candida glabrata's* Biofilms Oral Infections and Its Influence on the Matrix Composition and Gene Expression

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Abstract *Candida glabrata* is one of most prevalent yeast in fungal infections, especially in immunocompromised patients. Its azole resistance results in a low therapeutic response, particularly when associated with biofilms. The main goal of this work was to study the effectiveness of voriconazole (Vcz) against *C. glabrata* biofilms oral pathologies, as esophageal or oropharyngeal candidiasis. Antifungal susceptibilities were determined in pre-formed 24-h-biofilms and *ERG* genes expression was determined by qRT-PCR. Protein quantification was performed using BCA® Kit, carbohydrate was estimated according to the Dubois assay and β-1,3 glucans concentration were determined using Glucatell® kit. Finally, ergosterol, Vcz, and fluconazole (Flu) concentrations within the biofilm matrices were determined by RP-HPLC. Results showed that *C. glabrata* biofilms were more susceptible to Vcz than to Flu and that *ERG* genes expression evidenced an overexpression of the three *ERG* genes in the presence of both azoles. The matrix content presented a remarked decrease in proteins and an increase in carbohydrates, namely β-1,3 glucans. Ergosterol was successfully detected and quantified in the biofilm matrices, with no differences in all the considered conditions. Vcz demonstrated better diffusion through the biofilms and better cell penetration capacities, than Flu, indicating that the structure of the drug molecule fully influences its dissemination through the biofilm matrices. This work showed that Vcz is notably more effective than Flu for the treatment of resistant *C. glabrata* oral biofilms, which demonstrates a clinical relevance in its future use for the treatment of oropharyngeal/esophageal candidiasis caused by this species.

Keywords Candidiasis · Antifungal · Resistance · Matrix · Voriconazole · *Candida*

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Introduction

The occurrence of fungal infections, as candidiasis and candidemia, has been increasing significantly in the last decades, contributing to high morbidity and mortality. The enhanced use of broad-spectrum antibiotics, catheters and parenteral nutrition, the growth in the number of immunosuppressive diseases, the disruption of mucosal barriers, and the increase in chemotherapy and radiotherapy, are all important risk factors for the development of candidemia [1, 2]. *Candida* species belong to the normal microbiota of the individual's oral cavity, gastrointestinal, urinary, and vaginal tracts [2] and are responsible for numerous clinical manifestations

from mucocutaneous overgrowth to bloodstream severe infections [1]. *Candida albicans* is the predominant species in both health and disease conditions, yet, in the last two decades the number of infections due to non-*Candida albicans Candida* (NCAC) species increased significantly, particularly those involving *Candida glabrata.* This species lacks some of the virulence factors allied to *Candida* pathogenicity, such as hyphal growth or secret hydrolases [3], but it does not turn out to be less virulent [4, 5]. Oropharyngeal/esophageal candidiasis are serious oral diseases characterized by white patches or plaques (biofilms of *Candida*) on the tongue and other oral mucous membranes. These infections are usual among immunosuppressed adults, but can also be seen rarely in immunocompetent individuals [6–9].

The ability to form biofilm is one of the most important concerns in *Candida* pathogenicity. Biofilm production can occur on the host mucosa (e.g., mouth, esophagus) and on the surface of medical indwelling devices, involving a matrix that encloses yeasts' micro-colonies in a complex structure [4]. The antifungal biofilm resistance can be inducible in reaction to a drug, or a permanent genetic change resulting from prolonged exposure to that drug. Systematically, this phenomenon comprises changes or overexpression of the spot molecules, active extrusion through efflux pumps, limited diffusion, tolerance, which are all characterized mechanisms used by *Candida* to combat the antifungal treatment effects [4, 5].

Candida glabrata infections are characterized by an intrinsic low susceptibility to azoles, including the imidazoles (e.g., miconazole) and triazoles (e.g., fluconazole, voriconazole) [10, 11]. Voriconazole (Vcz) is a second-generation antifungal agent, which fortunately, presently shows a better *Candida* susceptibility [12–14]. This compound has shown to be a good alternative to treat candidiasis [15–19], in opposition to fluconazole (Flu), which has been largely used for decades, as a prophylactic or therapeutic treatment, inducing drug resistance mechanisms in *Candida* [10, 20].

The 2016's guidelines for esophageal and oropharyngeal candidiasis and patients having fluconazole-refractory disease recommend the use of voriconazole ("strong recommendation; high-quality evidence") [21]. Therefore, this work expects to elucidate the efficacy of Vcz in the treatment of these biofilm pathologies, specifically involving *Candida glabrata,* the most azole-resistant NCAC. It also aims at an evaluation of the response regarding the genes expression and matrix composition after a contact with this drug.

Methods

Organisms

Two clinical isolates of *C. glabrata* recovered from vaginal (*C. glabrata* 534784) and urinary (*C. glabrata* 562123) tracts, from Hospital Escala Braga in Portugal, and one reference strain from the American Type Culture Collection (*C. glabrata* ATCC2001) were used in the course of this study. 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 5.8 s subunit gene Ref. [22]. Genomic DNA was extracted following previously described procedures [23]. 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, Darmstadt, Germany) and incubated for 18 h at 37 °C under agitation at 120 rpm. After incubation, the cells were harvested by centrifugation at 3000*g* for 10 min at 4 °C and washed twice with phosphate-buffered saline (PBS, pH = 7.5). Pellets were then suspended in RPMI or SDA, and the cellular density was adjusted to 1×10^5 cells/mL, using a Neubauer counting chamber.

Antifungal Drugs

Vcz and Flu were kindly provided by Pfizer®, S.A. in its pure compound. Aliquots of 5000 mg/L were prepared using dimethyl-sulfoxide (DMSO). The final concentrations used were prepared with RPMI 1640 (Sigma-Aldrich, Roswell Park) or SDB medium and were: 10, 100, and 1000 mg/L for both drugs.

Biofilm Susceptibility

Standardized cell suspensions (200 µL) were placed into selected wells of 96-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). RPMI 1640 was used without cells and antifungal agent, as a negative control. As positive control, only cell suspensions were tested without antifungal agent. At 24 h, 100 µL of RPMI 1640 was removed and an equal volume of fresh RPMI 1640 plus the respective antifungal concentration were added (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 colony forming units (CFUs) [23]. 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.

Effect of Vcz and Flu on Biofilm Matrix

Extraction Method

For matrix analysis, biofilms were formed in 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) [23]. For this, 1000 µL of yeast cell suspension (1×10^5 cells/mL in SDB or SDB with Vcz or Flu at 10 and 1000 mg/L) were added to each well and the biofilms performed as described previously. After 24 h, 500 µL of SDB medium was removed and an equal volume of fresh SDB with or without the antifungal agents was added. After 48 h, biofilms were scraped from the 24-well plates, resuspended in ultra-pure water, sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W, and then the suspension vortexed for 2 min. The suspension was centrifuged at 5000*g* for 5 min at 4 °C and the supernatant filtered through a 0.2 µm nitrocellulose filter. The pellets were dried at 37 °C until a constant dry biofilm weight was determined.

Protein Determination

The protein content of the biofilm matrix was measured using the BCA® Kit (bicinchoninic acid, Sigma-Aldrich, St Louis, USA), using bovine serum albumin as a standard. The values were normalized per g of dry weight of biofilm and presented as mg of protein/g of dry weight of biofilm.

Carbohydrate Determination

Total carbohydrate content of the biofilm matrix was estimated according to the procedure of Dubois et al. [24], using glucose as standard. The values were normalized per g of dry weight biofilm and the values presented as mg of carbohydrate/g of dry weight of biofilm.

β-1,3 Glucans Concentration Determination

The β-1,3 glucans concentrations were determined using Glucatell® kit (Cape Cod, East Falmouth, USA). The values were normalized per pg/mL of β-1,3 glucans and per total of carbohydrates content.

Ergosterol Determination

For the ergosterol extraction, 2.0 mL of n-hexan (Fisher Chemicals) was added to 10.0 mL of the matrix suspension prepared as previously described and then submitted to vortex during 1 min. This procedure was performed three times and the top solution sequestered to a 10.0 mL amber bottle. After the extraction, the solutions were dried with nitrogen until all the organic solvent evaporated. The dried extract was resuspended in 2.0 mL of methanol (Fisher Chemicals), filtered with a 0.45 µm filter, and stored at −20 °C [25].

The high-pressure liquid chromatography (HPLC—Varian® 9002/Pro-Star) method was performed using a C18 column (YMC, Inc.). An isocratic mobile phase of 100% of methanol (Fisher Chemicals) with a flow of 1 mL/min, during 20 min was used for each sample quantification. The results were automatically revealed by the HPLC detector and then normalized by g of dry weight biofilm determined for each strain and condition [25].

Gene Expression Analysis

Gene Selection and Primer Design for Quantitative Real-Time PCR

Three genes (*ERG3*, *ERG6*, and *ERG11*) were selected to study their expression in biofilm cells in the absence and presence of both drugs. The gene sequences of interest were obtained from *Candida* Genome Database and the primers for quantitative real-time PCR (qRT-PCR) were designed using Primer 3 (http://primer3.ut.ee/) Web-based software and are listed in Table 1. *ACT1* was chosen to be the housekeeping gene. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from *C. glabrata* ATCC2001.

Preparation of Biofilm Cells for RNA Extraction

Biofilms of the three strains were grown in 24-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) in three different conditions—0 (control), 10 and 1000 mg/L of Vcz or Flu—with a final concentration of 1×10^5 cells/mL, as described above. After biofilm formation, the medium was aspired and the wells were washed with PBS to remove non-adherent cells. The biofilms were scraped from wells with 1 mL of PBS and sonicated (Ultrasonic Processor, Cole-Parmer, Illinois, USA) for 30 s at 30 W to separate the cells from the biofilm matrix. Cells were harvested by centrifugation at 5000 *g* for 5 min at 4 °C.

RNA Extraction

RNA extraction was performed using PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA). Prior to RNA extraction, a lysis buffer (PureLink RNA Mini kit) was prepared adding 1% of β-mercaptoethanol. Then, 500 µL of lysis buffer and glass beads (0.5 mm diameter) were added to each pellet. These mixes were homogenized twice for 30 s using a Mini-BeadBeater 8 (Stratech Scientific, Soham, UK). After cell disruption, the PureLink RNA Mini Kit (Invitrogen, Carlsbad, USA) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination samples were treated with RNase-Free I (Invitrogen, Carlsbad, USA).

Synthesis of cDNA

To synthesize the complementary DNA (cDNA) the iScript cDNA Synthesis Kit (Bio-Rad, Berkeley, USA) was used according to the manufacturer's instructions. For each sample 10 µL of the extracted RNA was used at a final reaction volume of 50 µL. cDNA synthesis was performed firstly at 70 °C for 5 min and then at 42 °C for 1 h. The reaction was stopped by heating for 5 min at 95 °C.

Quantitative Real-Time PCR (qRT-PCR)

Real-time PCR (CFX96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative levels of *ERG*3, *ERG*6, and *ERG11* mRNA transcripts in the RNA samples, with *ACT1* used as a reference for *Candida* housekeeping gene. Each reaction mixture consisted of a working concentration of SoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 50 µM forward and reverse primers, and 4 µL cDNA, in a final reaction volume of 20 µL. Negative controls (water), as well as, non-transcriptase reverse controls (NRT) were included in each run. The relative

Table 1 Primers and targets used for the gene expression analysis

Sequence (5′ → 3′)	Primer	Target
5′-CAC CCA GTC GGT TAC TT-3′	Forward	*ERG3*
5′-TTG ACA ACT GGG TTG GA-3′	Reverse	
5′-CTT CGA CAA AGT GTA CGC GA-3′	Forward	*ERG6*
5′-TAA ACG GCG AAA GTA CCA-3′	Reverse	
5′-CTC CAT ACT TGC CAT TCG-3′	Forward	*ERG11*
5′-CTT CAG TTG GGT AAC GCC AT-3′	Reverse	
5′-GTT GAC CGA GGC TCC AAT GA-3′	Forward	*ACT1*
5′-CAC CGT CAC CAG AGT CCA AA-3′	Reverse	

quantification of each gene expression was performed by the Pfaffl method [26].

Diffusion of Vcz and Flu Through the Biofilm Matrices

Sample Treatment

Biofilm was prepared as explained before, using a total volume of 500 µL. At 24 h, 250 µL were removed and an equal volume of fresh SDB plus the respective antifungal concentration (2 × concentrated) was added. The plates were incubated at 37 °C for more 24 h at 120 rpm. After 48 h, first, all medium was collected (approximately 350 µL), centrifuged at 12,000 rpm during 10 min, and filtered with a 0.45 µm filter. Then, 150 µL of sterile water was applied above the biofilm, for scraping it. The suspension was collected, sonicated during 10 s at 30 W, centrifuged at 12,000 rpm during 10 min, and finally filtered with a 0.45 µm filter. The samples were stored at −20 °C until the high-pressure liquid chromatography (HPLC) analysis [27].

Reverse-Phase High-Pressure Liquid Chromatography

The HPLC (HPLC—Varian 9002/Pro-Star) method was performed using a C18 column (YMC, Inc.) in a Varian 9002/Pro-Star equipment. An isocratic mobile phase was used for both drugs.

For Vcz, a slightly modified method from Ibrahim et al. [28] was used. The mobile phase was 0.05 M disodium hydrogen phosphate buffer (pH 5.5)/acetonitrile (1:1, v/v) (Fisher Chemicals) with a flow of 1 mL/min, during 10 min for each sample. The retention time was 7.5 min, at 255 nm.

For Flu, a slightly modified method from Shastri et al. [29] was used. The mobile phase was acetonitrile/water (50:50) (Fisher Chemicals) with a flow of 1 mL/min, during 6 min for each sample. The retention time was 3.5 min, at 260 nm.

As the diffusion procedure was developed and optimized by the group [27], all the controls and calibration curves were performed in order to guarantee its applicability.

The results were normalized by g of dry weight biofilm determined for each strain and condition.

All the described experiments were performed in triplicate and in three independent assays.

Statistical Analysis

Results were compared using one-way ANOVA and Dunnett's multiple comparisons test, using GraphPad Prism 5 software. All tests were performed with a confidence level of 95%.

Results

In order to determine the susceptibility of *C. glabrata* biofilms to Vcz, biofilms were developed during 24 h and then incubated for more 24 h in the presence of different concentrations of Vcz and Flu (control). Table 2 shows the percentage of cell death (biofilm cell viability) of each *C. glabrata* strain, when biofilms were exposed to 10, 100 and 1000 mg/L of Vcz and Flu concentrations. After a 10 or 100 mg/L treatment with the drugs, neither strains accomplished a reduction of, at least, 50%. On contrary, using 1000 mg/L of Vcz, *C. glabrata* 562123 reached a very good biofilm cell reduction of 70.57%, whereas *C. glabrata* ATCC2001 and *C. glabrata* 534784 reached 57.32% and 43.37% respectively. As expected, a different behavior occurred with Flu for all strains: this drug was unable to eradicate *C. glabrata's* viable biofilm cells, even using the higher concentration (1000 mg/

Table 2 Percentage of cell death found in biofilms of *C. glabrata* strains treated with different concentrations of voriconazole in comparison with cells exposed to fluconazole

C. glabrata strain	[Drugs] mg/L	% Biofilm cell death	
		Vcz	Flu
ATCC2001	10	0.62	0.75
	100	1.71	2.34
	1000	**57.32**	2.22
562123	10	0	0
	100	0	0
	1000	**70.57**	2.18
534784	10	0	3.54
	100	0	4.62
	1000	43.37	5.56

Bold: cell death higher than 50%

L) (Table 2). In fact, the higher reduction was noticed for *C. glabrata* 534784, merely 5.56%. This condition highlights the pronounced difference between a second-generation azole (Vcz) and a long-standing azole (Flu).

As it is well known, oral biofilms resistance to antifungal agents can be due to both cell genetic alterations and the presence of the biofilm matrix. Therefore, an evaluation of the influence of both agents in the expression of the genes (*ERG*) involved in the biosynthesis of ergosterol (the target of azoles), a well-known mechanism of azoles' resistance was ensuing performed [30, 31] and results are shown in Fig. 1. In general, the results demonstrate that the *ERG* gene expression levels are gene, strain, dose and azoles' molecule dependent, with significant differences ($P < 0.05$, $P < 0.001$ and $P < 0.0001$). *C. glabrata* ATCC2001, showed a visible lower n-fold expression for all *ERG* genes (Fig. 1) compared to the two isolate strains.

Regarding the study of the biofilm matrix composition and according to the previous results, *C. glabrata's* biofilms were treated with 10 and 1000 mg/L of Vcz and then compared with the control (Flu). In order to obtain additional insights into the causes of the increase of *C. glabrata's* oral biofilms resistance to azoles and the differences found between the two agents, the biofilm matrices were analyzed in terms of the most important compounds, namely total carbohydrates, β-1,3 glucans, proteins, and ergosterol (Fig. 2; Tables 2, 3).

Notably, a decrease was observed in protein quantities with an increase in the amount of carbohydrates, in the presence of both azoles. This pattern was specially noted for *C. glabrata* 562123 (Fig. 2). Overall, the quantity of carbohydrates in the presence of Vcz was a bit lower than in presence of Flu for the three strains. The quantification of proteins in biofilm matrices (Fig. 2) showed a significant decrease in biofilms grown with Vcz and Flu. The higher effect was observed for *C. glabrata* ATCC2001, with Flu application, and the lower effect for *C. glabrata* 534784, for both drugs. This effect was more perceptible for biofilms grown with Flu, especially for the lower concentration of antifungal agent tested (10 mg/L). Concerning the quantification of total β-1,3 glucans, the results confirm that, in general, there was an increase of total β-1,3 glucans per total carbohydrates, consistent with the previously observed rise of carbohydrates concentration.

Afterwards, the detection and quantification of ergosterol in biofilms matrices were carried out. This lipid was effectively detected but no alterations on its amount noticed in the matrices exposed to both agents (Table 4). For a final approach, the quantity of the azole agents present in the biofilm matrix and the consequent amount of drug capable of reaching the cells were also evaluated. A concentration of 1000 mg/L of each azole was applied in a 24-h-biofilm for an additional 24 h. Then, the quantity diffused within the biofilm and/or absorbed by the cells was determined according to a protocol described for Flu [27]. Interestingly, the results indicate that Vcz had a higher capacity to penetrate the biofilm net, reaching and eliminating the cells. As it can be observed in Table 4, Flu was not so effective in all these processes, which may explain its inability to reduce *C. glabrata's* oral biofilms, on contrary to the good therapeutic response that Vcz has shown (Table 4).

Discussion

Among the *Candida* species, *C. glabrata* is the second most prevalent pathogen in humans in the USA and the third in Europe, after *C. albicans* and *C. parapsilosis* [32, 33]. *Candida* species developed the capability to adapt to different niches and to invade quite a lot of epithelia, namely esophagus, oropharynx or, in even more serious cases, causing septicemia. These infections are very difficult to treat with the traditional antifungal therapies and, consequently, have a high reported morbidity and mortality [34–38].

Agents within the azole class, like Vcz and Flu, are able to vary decisively affecting the spectrum of activity, their pharmacokinetic profiles, and toxicities. Vcz and Flu are triazolic derivatives, resulting from replacement of the imidazole ring by triazole, which favors a broad spectrum of action and selectivity for cytochrome P450 fungal cell. *Candida glabrata* grows only as yeast form in vivo, and it is believed that the increasing of *C. glabrata* infections is due to its intrinsically low susceptibility to azoles (e.g. Flu) [39–41].

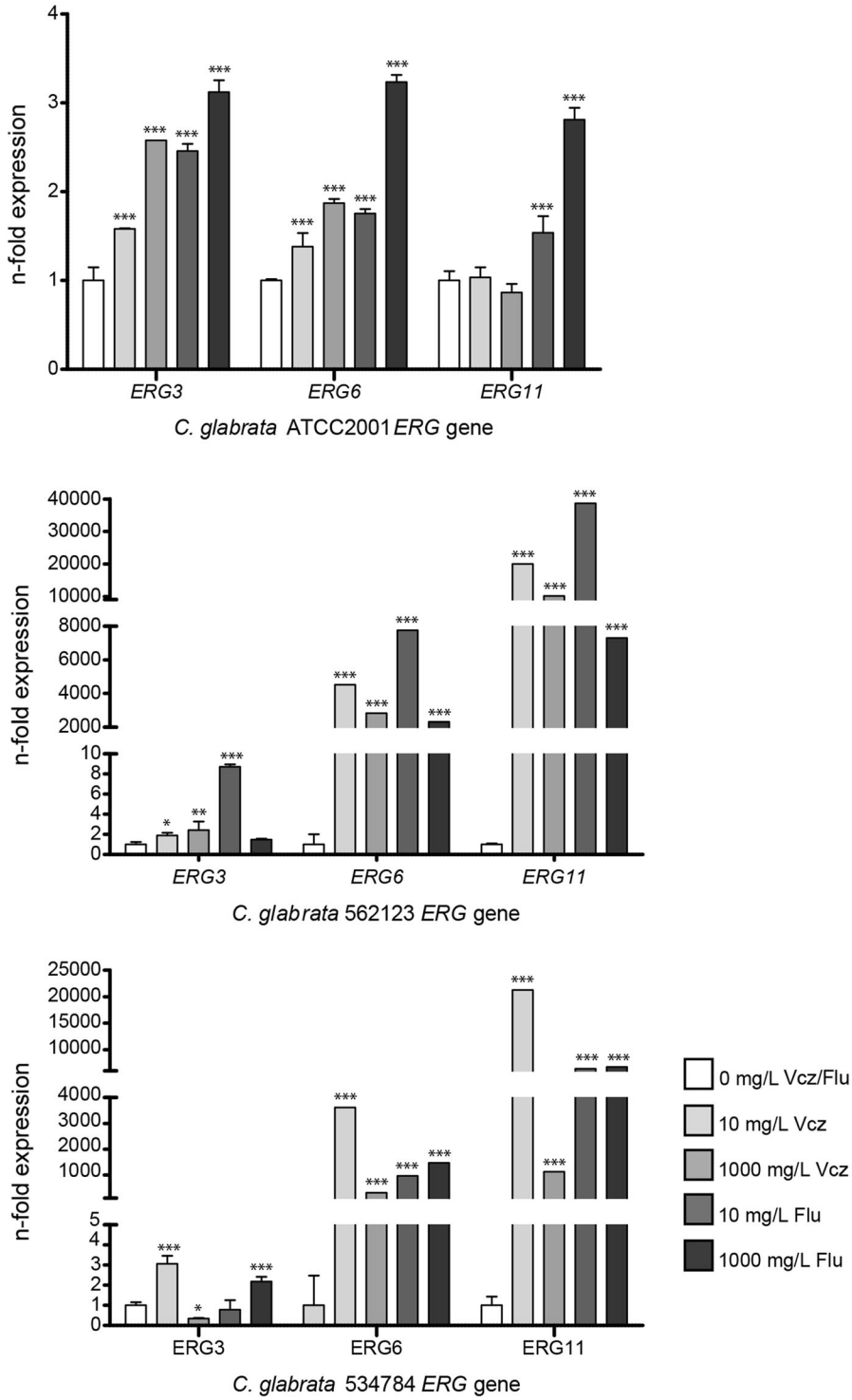

Fig. 1 *ERG*3, *ERG*6, and *ERG*11 *n*-fold expressions in biofilm cells for each strain, with different concentrations of voriconazole and fluconazole (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$)

Therefore, the main purposes of this work were to evaluate if Vcz is a good choice to treat, specifically, *C. glabratás* oral biofilms infections, as well to elucidate the mechanisms behind its different responses. Due to its recurrent and traditional use, Flu was used as a control during this study.

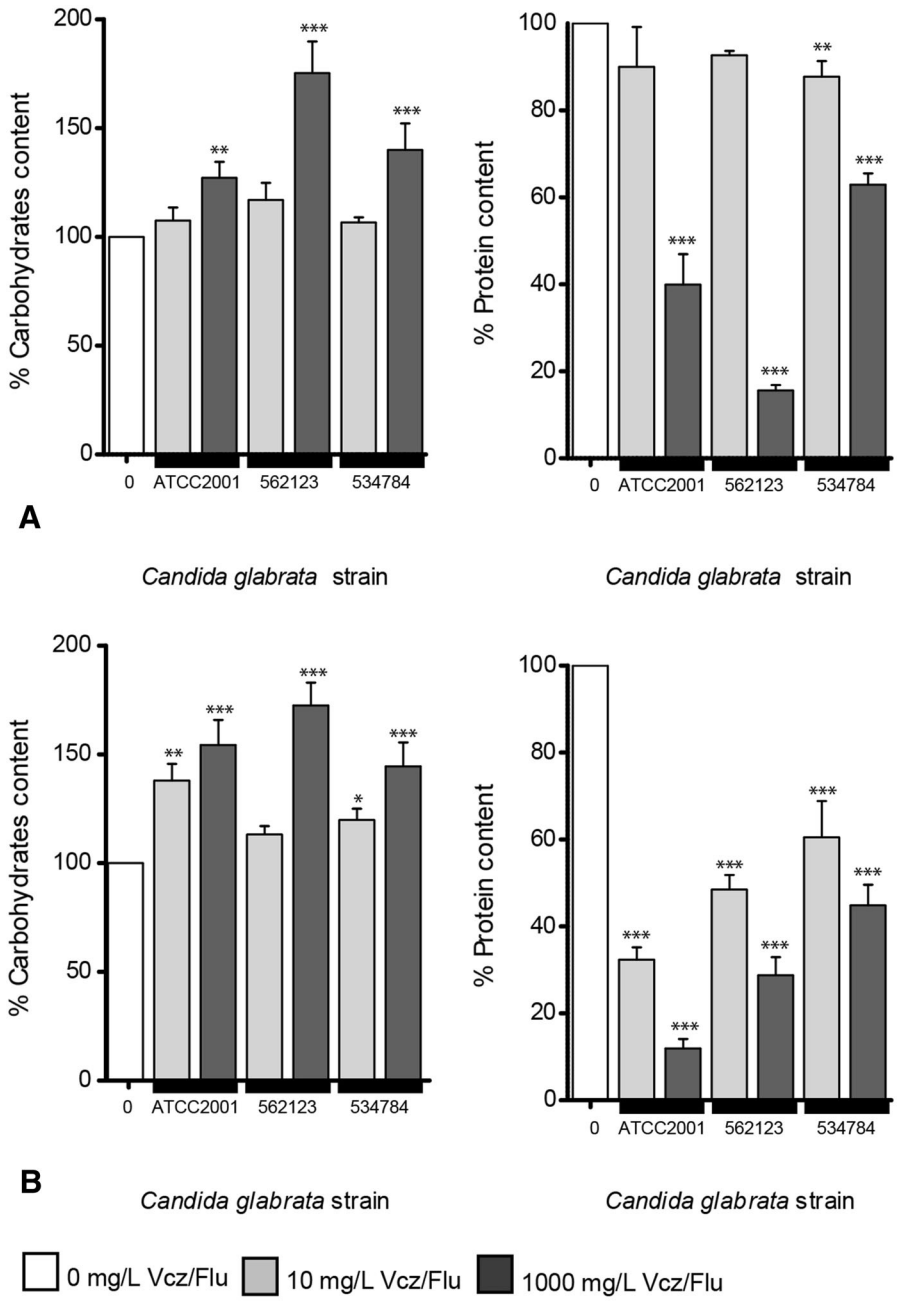

Fig. 2 Content of carbohydrates and proteins content in biofilm matrices of *Candida glabrata* strains treated with different concentrations of voriconazole (**A**) and fluconazole (**B**) (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$)

Regarding the susceptibility tests of *C. glabrata's* biofilms, results show that the two lower concentrations (10 and 100 mg/L) were not effective for the reduction of a minimum of 50% of the biofilm for both drugs. The same happened with 1000 mg/L of Flu. In opposition, with 1000 mg/L of Vcz, this reduction was easily reached, noticeably for *C. glabrata* 562123 which had the highest decrease (70.57%). These evidences corroborate the reported facts that (oral) biofilm cells are much more resistant to antifungal agents than planktonic cells [42–47] and are particularly resistant to azoles [48–50].

The relation between the role of the *ERG3*, *ERG6*, and *ERG11* expression with the Vcz biofilm's cells susceptibility was evaluated in *C. glabrata's* biofilm grown in the presence of Vcz or Flu, by qRT-PCR

Table 3 β-1,3 glucans/carbohydrates ratio and ergosterol concentration on biofilm matrices of *Candida glabrata* strains in the presence of voriconazole and fluconazole

C. glabrata strain	[Drug] mg/L	[β-1,3 glucans]/carbohydrates ratio Vcz	[β-1,3 glucans]/carbohydrates ratio Flu	[Ergosterol] mg/g biofilm ± SD Vcz	[Ergosterol] mg/g biofilm ± SD Flu
ATCC2001	0	1.2×10^{-4}		1.17 ± 0.70	
	10	1.8×10^{-4}	9.8×10^{-4}	0.58 ± 0.21	1.15 ± 0.77
	1000	1.1×10^{-4}	7.4×10^{-4}	0.79 ± 0.16	0.51 ± 0.14
562123	0	1.3×10^{-4}		1.48 ± 0.71	
	10	1.7×10^{-4}	8.8×10^{-4}	0.95 ± 0.47	1.01 ± 0.09
	1000	9.0×10^{-5}	4.3×10^{-4}	1.45 ± 0.30	1.39 ± 0.25
534784	0	1.2×10^{-4}		1.32 ± 0.67	
	10	2.7×10^{-4}	11.4×10^{-4}	1.04 ± 0.21	0.75 ± 0.05
	1000	1.7×10^{-4}	7.5×10^{-4}	2.16 ± 1.01	1.45 ± 0.07

SD standard deviation

Table 4 Antifungal drug diffusion through *Candida glabrata* biofilm matrices and supernatant

Drug	*C. glabrata* strain	[Drug] matrix (mg/L) ± SD	[Drug] supernatant (mg/L) ± SD
Voriconazole	ATCC2001	0.44 ± 0.20	5.38 ± 1.20
	562123	0.43 ± 0.21	5.27 ± 1.38
	534784	0.60 ± 0.25	5.85 ± 1.67
Fluconazole	ATCC2001	670.80 ± 61.87	69.21 ± 21.49
	562123	551.87 ± 56.31	60.81 ± 20.10
	534784	702.29 ± 77.92	69.88 ± 17.06

SD standard deviation

(Fig. 1). *ERG3,* the gene responsible for the conversion of ergosta-5, 7, 24 (28)-trienol to episterol [51], showed an inferior expression for all strains in the presence of Vcz and Flu. *ERG6*, linked to the transformation of fecosterol to zymosterol and *ERG11*, the gene in charge of the production of lanosterol from ignosterol (ergosterol biosynthesis), presented the greater expression in both azoles, remarkably for the isolate strains (*C. glabrata* 562123 and *C. glabrata* 534784). This event occurred probably due to the interference of the azole drugs with the 14α-lanosterol demethylase, obstructing its pathway to produce ergosterol, failing to repose this lipid in the cell membrane [51]. In general, the response was higher for lower concentrations of both drugs (10 mg/L). This assertive reaction to the presence of Vcz and Flu shows the high ability of *C. glabrata* to rapidly adapt to stress, as it has been showed before [52–55], but does not fully explain the response of *C. glabrata* cells when Vcz is used (and comparing with Flu) in clinical therapeutics. So, the absence of a clear correlation between *ERG* genes expression and the susceptibilities profiles led us to explore the role of the biofilm matrix in azole resistance.

Figure 2 shows a solid decrease in the protein quantification in the presence of both azoles, especially for *C. glabrata* ATCC2001, with the use of Flu (and mostly in the lower concentration used), a phenomenon that may be explained by a response in the stress induced by the drug [56]. On the other side, both drugs instigated a minor effect in the loss of proteins, for *C. glabrata* 534784. On the contrary, biofilms developed with Vcz or Flu displayed an increase in the amount of carbohydrates (Fig. 2), increase that was also confirmed in the quantification of β-1,3 glucans (Table 3). β-1,3 glucans are found in cell wall of *Candida* species and also in the biofilm

matrix, being linked to the antifungal resistance, by making it difficult to the drugs to diffuse through the biofilm matrices and reach the yeast cells [57–59]. This change in the biofilm matrix composition may be an effort of the cells to obtain a denser matrix, inflexible and more protective for their environment with these biopolymers. Thus, for the obtained results, it is likely that *C. glabrata's* biofilm cells are attempting to respond to the azole-caused-stress, producing as many polymers of carbohydrates as possible, aiming to thick the biofilm matrix and, thus, protecting the cells against the drugs.

Additionally, it was questioned whether ergosterol, which is a specific *Candida* cell membrane lipid, could be an important factor in the antifungal resistance or *C. glabrata's* oral biofilm infections. The results showed that this lipid could not be associated with the resistance or tolerance mechanisms, since, generally, there were no significant variations on its quantity in the biofilm matrices exposed to Vcz or Flu (Table 3).

Ultimately, the determination of the quantity of the azole agents present in the biofilm matrix and the consequent amount of drug capable of reaching the cells confirmed that Vcz has the capacity to infiltrate better the biofilm matrices than Flu (Table 4). As it is known, Flu has a chemical structure distinct from other antifungal drugs, providing a pharmacokinetic profile highly differentiated with a molecular mass of 306.27 g/mol. The Vcz chemical structure, although similar to Flu, presents a replacement of one triazole group for a fluoropyrimidine and an introduction of a methyl group and a molecular mass of 349.31 g/mol. This univalent methyl group shows a stronger hydrophobic interaction with aromatic amino acids and more extensive filling of the substrate-binding site [30], making Vcz more absorbable (96%) than Flu (90%) and with a better connection to the fungi cells, thus emerging as a standard treatment for oropharyngeal or esophageal candidiasis [21]. This was in fact substantiated in this work where *C. glabrata* biofilms are much more susceptible to Vcz than to Flu (Table 4).

Concluding, this study showed that the pattern of *C. glabrata's* biofilms susceptibilities to Vcz and its comparative molecule, Flu, are effectively different. As it is known, one of the possible mechanisms of resistance to both agents is cell genetic alterations in genes encoding for the ergosterol biosynthesis, which seems to be also the key for the high levels of *C. glabrata* biofilm azoles resistance, but this fact was unable to completely explain the different resistance profiles obtained for these two agents. Although there were alterations in the matrix composition (proteins, carbohydrates, including β-1,3 glucans, and ergosterol) in the presence of the azoles, there was not found a direct correlation with Vcz efficacy.

Still, this study demonstrated that the diffusion of the drugs within the biofilms is dependent on the azole used, with Vcz having a better diffusion and an enhanced activity against *C. glabrata* biofilms than Flu, explaining, in part, its better performance and efficacy for oral candidiasis.

This study also remarks that the tolerance or resistance mechanisms to azoles are multifactorial and cannot be linked to only one feature associated specifically to the cells, the environment or the ecosystems where they persist.

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Compliance with Ethical Standards

Conflict of interest All the authors declare that there is no financial/personal interest or belief that could affect their objectivity.

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