The Microbe 3 (2024) 100062

Contents lists available at ScienceDirect

The Microbe

journal homepage: www.sciencedirect.com/journal/the-microbe

2-aryloxazolines inhibit *Candida* clinical isolates growth and morphogenesis of *Candida albicans* and *Candida tropicalis*

Vinícius M. Barroso [a], Joel S. Reis [b], Hélio A. Stefani [c], Kelly Ishida [a,*]

[a] Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[b] Institute of Chemistry, Fluminense Federal University, Niterói, Brazil
[c] School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil

ARTICLE INFO

Keywords:
2-aryloxazolines
Candida albicans
Candida tropicalis
Morphogenesis
Virulence factors

ABSTRACT

New antifungal molecules are being researched addressing new targets and mechanisms of action. Therefore, the present study aimed to evaluate the antifungal activity of 2-aryloxazoline derivatives (**4i** and **9i**) on *Candida* clinical isolates and on morphogenesis of competent-filament species. Both compounds inhibited *Candida* spp. growth at concentrations ≤0.03–2 μg/mL, including less susceptible and resistant isolates to standard antifungals. However, neither fungicidal effect of compounds nor synergistic/antagonistic interactions in combination with antifungals were observed. On the other hand, *C. albicans* treated with 2-aryloxazoline exhibited some ultrastructural changes such as the integrity loss of the plasma membrane and cell wall. In *C. albicans*, the proteinase activity was reduced after treatment with high concentrations of 2-aryloxazoline, but this inhibition was not observed in *C. tropicalis*. The compounds inhibited up to 50% of the total biomass of *C. albicans* and *C. tropicalis* biofilms but did not inhibit the metabolic activity. Additionally, 2-aryloxazolines caused a dose-dependent reduction in the pseudohyphae/hyphae formation of both *Candida* species (*C. albicans* and *C. tropicalis*) in RPMI medium and other filament-inducing media. Notably, the compounds caused a total inhibition of pseudohyphae/hyphae formation in Spider broth, a partial inhibition in Lee medium, and no inhibition in SM supplemented with N-acetylglucosamine. This effect on the yeast-hyphae transition was associated with blockage of cAMP and MAPK pathways due to the gene downregulation of the transcription factors *TEC1*, *EFG1,* and *CEK1* in 4i-treated *C. albicans* cells. In this regard, the adhesins (*HWP1* and *ALS3*) and candidalysin (*ECE1*) genes were also downregulated as a result of the interference of compounds on the filamentation signaling pathways. Therefore, 2-aryloxazolines showed promising results inhibiting *Candida* growth and morphogenesis; however, other approaches should be carried out to better elucidate other possible mechanisms of action.

1. Introduction

Invasive fungal infections by *Candida* species emerged as one of the main causes of infectious diseases in humans becoming a worldwide health problem, affecting hospitalized patients who are debilitated or immunocompromised (Bongomin et al., 2017; Lass-Florl et al., 2024). *Candida albicans* is the main species associated to candidemia, but other species have gained notoriety such as *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* (now named *Nakaseomyces glabratus)*, *Candida krusei* (now named *Pichia kudriavzevii),* and *Candida auris* (Ramos et al., 2015; Sears and Schwartz, 2017; Bongomin et al., 2017; Lass-Florl et al., 2024). Mortality rates in patients with candidemia are high ranging from 40% to greater than 70% depending on the severity of the patient, even with antifungal treatment (Rodriguez et al., 2017; Salci et al., 2018; Yap et al., 2009; Lass-Florl et al., 2024). Recently, the World Health Organization classified some species of *Candida* as critical and high priority pathogens to guide research, development, and public health action (World Health Organization WHO., 2022).

Polyenes, azoles and echinocandins are recommended antifungals in the treatment of invasive candidiasis and candidemia (Pappas et al., 2016). However, they have some limitations, as narrow spectrum of action, high toxicity, drug-drug interactions, low chemical stability,

* Correspondence to: Laboratory of Antifungal Chemotherapy, Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, Prof. Lineu Prestes Avenue, 1374 – 05508-000, ICB II, Lab 150, São Paulo, SP, Brazil.
E-mail addresses: viniciusmbmb@gmail.com (V.M. Barroso), joelreis@id.uff.br (J.S. Reis), hstefani@usp.br (H.A. Stefani), ishidakelly@usp.br (K. Ishida).

https://doi.org/10.1016/j.microb.2024.100062
Received 24 November 2023; Received in revised form 17 January 2024; Accepted 22 March 2024
Available online 23 March 2024
2950-1946/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

poor water solubility, and reduced gastrointestinal absorption leading a low oral bioavailability (Pappas et al., 2016). Moreover, there are increasing and emerging isolates resistant to more than two antifungals (Perlin et al., 2017) and the emergence of intrinsically resistant species as *C. auris* (Arendrup and Patterson, 2017). Thus, the search for new compounds is needed to solve the lack of options to current antifungals, ideally molecules from a new chemical class and different mechanisms of action.

Candida species are often found in oral cavity and gastrointestinal tract as commensal, but they have developed specific strategies to infect and invade the tissues of susceptible hosts (Naglik et al., 2003). Among the virulence factors, adherence, biofilm formation, production of hydrolytic enzymes and toxins, and mechanisms of stress tolerance are the most relevant, and the transition from yeast to hyphae/pseudohyphae is associated to fungal pathogenesis of some species as *C. albicans* and *C. tropicalis*. In this way, in addition to inhibiting the growth of *Candida* species by novel compounds, e.g., fosmanogepix, ibrexafungerp, filastatin, and others, they also inhibit the expression of virulence factors including yeast-hyphae transition and infection using *in vitro* and *in vivo* models (Kalimuthu et al., 2022; Murphy and Bicanic, 2021; Pierce et al., 2015).

Our research group demonstrated in previous work that 2-aryloxazoline derivatives, mainly the compounds **4i** and **9i**, inhibited the growth of ten *Candida* spp., including fluconazole-resistant isolates, and had low cytotoxicity on HepG2 cells reflecting in high selectivity indexes (Argomedo et al., 2020). In this regard, we investigated here the antifungal action of 2-aryloxazolines against *Candida* spp. clinical isolates and their effects on virulence factors, especially on the morphogenesis of *C. albicans* and *C. tropicalis*.

2. Material and methods

2.1. Strains

Sixty-nine strains of *Candida albicans* and *Candida* non-*albicans* (10 standard strains and 61 clinical isolates previously described (Muñoz et al., 2017)) were included in this work: *C. albicans* (n=16), *C. parapsilosis* (n=15), *C. tropicalis* (n=12), *C. glabrata* (n=13), *C. krusei* (n=10), and *C. guillermondii* (n=3) (See Table S1). The strains are stored in Brain Hearth Infusion broth (Becton, Dickinson and Company, USA) and 20 % glycerol at −80 ºC. Yeasts were recovered in Sabouraud dextrose agar (SDA; Kasvi, Brazil), the pure culture was confirmed on Chromagar *Candida* (Becton, Dickinson and Company, USA), and they were subcultured on SDA at least twice at 35 ºC for 24 h prior to assays.

2.2. Standard antifungal

Fluconazole (FLC), voriconazole (VRC), caspofungin (CAS), and micafungin (MFG) were purchased from Sigma-Merck. The antifungals were dissolved in dimethylsulfoxide (DMSO, Vetec) to obtain a stock solution at least 100 x final concentration used in the assays and kept at −20 ºC.

2.3. Synthesis of 2-aryloxazoline compounds

The synthesis of 2-aryloxazolines **4i** and **9i** (Fig. 1) was performed from the reaction between L-threonine and derivatives of salicylic or naphthoic acids, respectively, as previously published (Argomedo et al., 2020).

2.4. Antifungal activity of 2-aryloxazolines on Candida spp. clinical isolates

Candida spp. clinical isolates and standard strains (n=69) were used to extend the *in vitro* inhibitory activity data of the 2-aryloxazoline derivatives using the broth microdilution method (Clinical Laboratory Standard Institute CLSI., 2017). Briefly, **4i**, **9i** and antifungals (FLC, VRC, CAS, and MFG) were serially diluted 1:2 in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich, Massachusetts, USA) buffered with 0.165 M 3-(N-morpholino) propanesulfonic acid (Sigma-Aldrich, Massachusetts, USA) and pH 7.0 (or simply RPMI) in 96-well flat-bottomed polystyrene plates. Yeast suspension was dispensed into each well to obtain final concentrations of 0.5–2.5 $\times 10^3$ CFU/mL and 0.008–16 μg/mL of **4i**, **9i** or antifungals, except to FLC (0.125–64 μg/mL). Wells containing only medium and yeasts and only medium were included as positive and negative fungal growth controls, respectively. *Candida parapsilosis* ATCC 22019 was used as a quality control strain of the assay.

The plates were incubated for 24 h at 35 °C to determine the minimum inhibitory concentration (MIC) of **4i**, **9i** and antifungals, defined as the lowest concentration that inhibits 50% of fungal growth by visual inspection. The interpretation criteria, i.e., the breakpoint values for FLC, VRC, CAS and MFG were based on CLSI document (Clinical Laboratory Standard Institute CLSI., 2022). After that, MIC_{50} and MIC_{90} values were determined, as possible, and they were defined as the concentrations that inhibit 50% and 90% of all tested strains, respectively.

After MIC readings, 10 μL-aliquot of all concentrations that inhibited the fungal growth was cultured on drug-free SDA medium for 24 h at 35 ºC to determine the minimum fungicidal concentration (MFC) defined as the lowest concentration of the compounds and antifungals that kills > 99.9% of initial inoculum (Cánton et al., 2003).

After susceptibility testing of compounds **4i** and **9i** against clinical isolates, representative strains of *Candida* species that form true hyphae *C. albicans* and *C. tropicalis* were selected for the next assays to evaluate the antifungal combination and effects on budding, proteinase activity, biofilm formation, cell ultrastructure, and yeast-hyphae transition. For some experiments, only compound **4i** was used due to the higher synthesis yield, in addition to molecule **9i** being a structural analogue of **4i** (Argomedo et al., 2020).

2.5. Combination of 2-aryloxazolines with standard antifungals

The combinations of **4i** or **9i** with standard antifungal (AMB, FLC or MFG) diluted in RPMI were performed by the checkerboard method (Mukherjee et al., 2005). After incubation for 24 h at 35 °C the fractional inhibitory concentration index (FICi) was determined to evaluate the

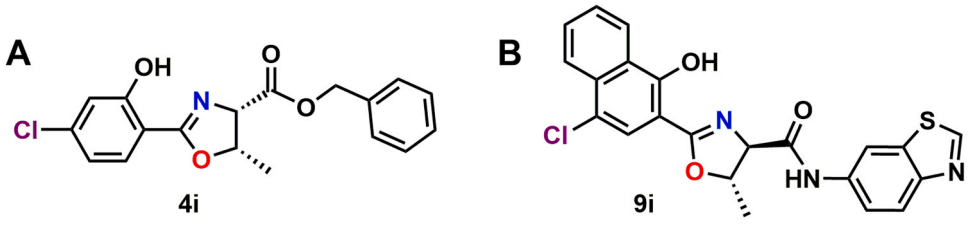

Fig. 1. Molecular structures of 2-aryloxazolines derivatives. **(A) compound 4i** benzyl (4*S*,5 *S*)-2-(4-chloro-2-hydroxyphenyl)-5-methyl-4,5-dihydrooxazole-4-carboxylate. **(B) compound 9i** (4 *R*,5 *S*)-N-(benzo[*d*]thiazol-6-yl)-2-(4-chloro-1-hydroxynaphthalen-2-yl)-5-methyl-4,5-dihydrooxazole-4-carboxamide.

interaction according to the following criteria: FICi ≤0.5, synergistic action, FICi > 0.5 and ≤ 4, indifferent, and FICi > 4, antagonistic action (Muherjee et al., 2005).

2.6. Effect of 2-aryloxazolines on budding, proteinase activity and biofilms of Candida spp.

Budding quantification: After broth microdilution assay, C. *albicans* SC 5314 and *C. tropicalis* IAL-01 treated or not with compounds **4i** and **9i** at MIC values were visualized in a light microscope for counting of yeasts with and without budding using a Neubauer chamber (~150 cells) for budding percentage determination.

Proteinase assay: **4i** and **9i** were added to bovine serum albumin (BSA) agar (2 g BSA fraction V, 6.7 g yeast nitrogen base w/o amino acids, 1.45 g ammonium sulfate, 20 g glucose, 20 g agar, and 1000 mL distilled water) to obtain the final concentrations of 2–64 μg/mL to assess the inhibitory activity on fungal proteinase activity. A volume of 10 μL of yeasts of C. *albicans* SC 5314 or *C. tropicalis* IAL-01 at 1×10^6 CFU/mL in PBS was aliquoted onto the surface of BSA agar, incubated at 35 ºC for 72 h. The proteinase production was detected by the degradation halo around the colony and the enzyme activity (Pz) was determined by the ratio between the diameter of the degradation zone around the colony and the colony diameter (Mardegan et al., 2006).

Biofilm formation: An aliquot of 100 μL of C. *albicans* SC 5314 or *C. tropicalis* IAL-01 at 1×10^6 CFU/mL in RPMI was dispensed into the wells of a 96-well flat-bottomed polystyrene plate and incubated at 35 °C for 1.5 h, under agitation (150 rpm). After the adhesion period, the supernatant was discarded and 100 μL of different concentrations of compounds **4i**, **9i** or FLC (16–128 g/mL) were diluted in RPMI and dispensed into each well and the plate incubated for 24 h at 35 °C, under agitation (150 rpm). After, the supernatant was discarded for evaluation of metabolic activity of sessile cells by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2 H-tetrazolium hydroxide (XTT) reduction assay (Sigma-Aldrich, Massachusetts, United States) (Freitas et al., 2018). In addition, the total biomass of biofilm treated with **4i** was measured after incubation periods of 4, 8, 12 and 24 h using violet crystal stain (Freitas et al., 2018).

2.7. Evaluation of morphogenesis of Candida albicans and Candida tropicalis treated with 2-aryloxazoline derivatives

Yeats of *C. albicans* (SC 5314, ATCC 10231 and IAL-40 [FLC-resistant]) and *C. tropicalis* (IAL-01 and IAL-04 [FLC-resistant]) were obtained after culture on SDA at 30 ºC for 24 h. Then, yeasts were treated with concentrations of 0.125–16 μg/mL of **4i** or **9i**, for 24 h at 35 ºC, in different hyphae-inducing media to determine the MIC values (Clinical Laboratory Standard Institute CLSI., 2017). The hyphae-inducing media were Spider broth (Efg1 pathway), Lee medium (Cph2 and Tec1 pathways), and SM medium supplemented with N-acetyl-glucosamine (SM-GlcNAc) (activates Ngt1 signal) (Castilla et al., 1998; Sudbery, 2011; Vila et al., 2017) and the MIC data were compared with treatment in RPMI (induces multiple pathways) (Romo et al., 2017).

After MIC determination, yeast, budding, pseudohyphae, and true hyphae were observed in a light microscope for qualitative and semi-quantitative analysis of these morphological structures. In addition, images from light and fluorescence microscopy after labeling with Calcofluor White M2R (1 mg/mL, Sigma-Aldrich, Massachusetts, USA) were captured (EVOS FL, Thermo Fisher Scientific, Massachusetts, USA).

2.8. Effect of 2-aryloxazolines on the virulence genes expression of Candida albicans

Virulence genes associated with the filamentous phase in *C. albicans* SC5314 were chosen for analysis of their expression: *SAP1, SAP6, ECE1, RIM8, CEK1, RAS1, CPH1, CPH2, TEC1, NGT1, CYR1, ALS3, HWP1*, and *EFG1* (Table 1). Beta actin (*ACT1*) was used as a reference gene in the expression of the virulence genes. The forward and reverse primers were designed in the Primer3plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) using genome sequences from *C. albicans* SC5314 strain available in the GenBank database (https://www.ncbi.nlm.nih.gov), except for *ALS3, HWP1*, and *EFG1* which were obtained from previous works (Alonso et al., 2018; Alves et al., 2014).

Yeasts at 1×10^6 CFU/mL untreated and treated with MIC of **4i** in RPMI medium for 24 h at 35 ºC were analyzed for expression of virulence genes. After incubation, the total RNA was extracted with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany), quantified using Nanodrop (Thermo Fisher, Massachusetts, United States) and an absorption rate at 260/280 nm between 1.8 and 2 indicated an acceptable RNA quality. Subsequently, cDNA was obtained using the MMLV Rnase H minus first strand cDna synthesis kit (Nova biotechnology, Brazil) and the qPCR reaction was performed using the SYBR Green QPCRMaster Mix Low Rox kit (Nova biotechnology, Brazil). The validation of the gene expression was performed with a relative standard curve in the method comparative CT and calculate arithmetic formula $2^{-\Delta\Delta CT}$.

2.9. Transmission electron microscopy

Yeast of *C. albicans* (SC5314) treated with MIC of **4i** in RPMI medium for 24 h at 35 ºC were collected by centrifugation and washed three times in PBS. After, cells were fixed for 2 h with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, post-fixed in 1 % osmium tetroxide in cacodylate buffer containing 1.25 % potassium ferrocyanide and 5 mM $CaCl_2$ for 2 h at room temperature. The cells were dehydrated in

Table 1
Primers used for virulence factors expression of *Candida albicans*.

genes	Sequence 5′ 3′	
	Forward	**Reverse**
EFG1	ACGAGTAACAACTACCAT	TATCTGCTCTTCTGACAA
ALS3	CTGGACCACCAGGAAACACT	GGTGGAGCGGTGACAGTAGT
HWP1	TCTACTGCTCCAGCCACTGA	CCAGCAGGAATTGTTTCCAT
SAP1	CTCGTCCTGGTCAATCAGCA	GCACCACCAAAACCAACAGT
RAS1	TTGTTGGAGGTGGTGGTGTT	TGGCCAGATATTCTTCTTGTCCA
CPH1	TGCCGCCAATTTCAGCAAAA	ACAGCACCGGTATTTCTGCT
CPH2	ACAGGAAGTTTAAGCCACAATGAAG	GTGGGAAGTCACCTTGTTTGTTTAT
TEC1	ATGCATCTCCCAGCCACAAA	ACGTCATTGCCATTTTGCTGT
NGT1	CCGGCTCGTTGTTGTCATTC	TGAACCAATGACTGCACCCA
CYR1	CATGGTTGCCTTTCCTTCGC	TCACATACAGGAGAGCCCCA
SAP6	TGGTGGTATTGACAAGGCCA	AGGACACCAGCGTTGACATT
ECE1	ATTGTTGCTCGTGTTGCCAC	CCAGGACGCCATCAAAAACG
RIM8	AACCCGCAAGCAACCAAATC	GTGGTGCTTGCTCTTCTTGC
CEK1	TACTACTTCTTCTTCCCCTCGTCAA	TGGCTGAACAAACTATTCCATATGC
ACT1	GTTTTGTTGACCGAAGCTCCAA	CGTGAGTAACACCATCACCAGA

increasing ethanol concentrations, then placed into 100 % propylene oxide and embedded in spur's resin. Ultrathin sections were stained with 0.5 % uranyl acetate and 0.5 % lead citrate, and observed in a transmission electron microscope (FEI, Japan).

2.10. Statistical analysis

The statistical analyses were performed by the GraphPad Prism v.8.0 (GraphPad Software Inc.) and a *P*-value less than 0.05 was considered significant. MIC values were analyzed by linear regression test (Pearson correlation) and student's t test (paired t test). Differences in the budding percentage, proteinase activity and biofilm total biomass of untreated and treated *Candida* were analyzed by One way ANOVA (Dunnett test).

3. Results

3.1. 2-aryloxazoline compounds inhibit Candida clinical isolates growth

Eleven isolates resistant to FLC [*C. albicans* (IAL-40 and IAL-42), *C. parapsilosis* (IAL-15, IAL-17, and IAL-18), *C. tropicalis* (ATCC 200956, ATCC 28707, IAL-04, IAL-8, and IAL-10) and *C. glabrata* (IAL-23)] in addition to FLC intrinsic resistance of *C. krusei* (n=10) were observed in this work (Table S1) and in previous published work (Freitas et al., 2018). All clinical isolates were susceptible to VRC and echinocandins CAS and MFG, except *C. krusei* IAL-30 classified as resistant to MFG and 4 isolates had intermediary susceptibility to VRC (*C. albicans* IAL-40 and IAL-56, *C. tropicalis* IAL-8 and *C. parapsilosis* IAL-18) (Table 2 and S1).

The compounds **4i** and **9i** showed inhibitory effect on *Candida* spp. growth at MICs ranging from 0.03 to 2 μg/mL and the MIC_{50} and MIC_{90} values were lower than 0.5 μg/mL. It is important to highlight that both compounds inhibited the fungal growth of isolates resistant or less susceptible to standard antifungals (Tables S1 and S2). However, both compounds had a fungistatic effect that MFC values were >16 μg/mL as well as the standard antifungal FLC (Table 2 and S2).

A high positive correlation of MIC values of compounds **4i** and **9i** was observed ($r=0.89$, $p<0.0001$) while no significant correlation was found between 2-aryloxazolines with azoles (FLC or VRC) and echinocandins (CAS or MFG). In addition, a significant lower MIC values was considered for **9i** when compared with its structural analogue **4i** ($p<0.0001$) and no significant difference was observed between MIC values of **9i** and standard antifungals VRC, CAS, and MFG ($p>0.05$). Although **4i** had higher MIC values compared with VRC and echinocandins, its inhibitory activity was better than FLC (Tables S1 and S2).

In addition, all combinations of compounds **4i** or **9i** with antifungals (AMB, FLC, or MFG) were indifferent that FICi were >0.5 and ≤4 (Table S3).

Table 2
Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of 2-aryloxazolines (**4i** and **9i**) and standard antifungals against *Candida* spp. strains.

Strains	Concentrations (μg/mL)					
	MIC values	4i	9i	VRC[a,b]	CAS	MFG[c]
C. albicans **(n=16)**	MIC range	0.062–2	0.031–1	0.031–0.5	0.031–0.25	≤0.008–1
	MIC average	0.4	0.24	0.1	0.085	≤0.008
	MIC_{50}	0.25	0.125	0.031	0.031	≤0.008
	MIC_{90}	0.5	0.5	0.25	0.25	≤0.008
	MFC range	>16	>16	>16	0.06->16	0.008->4
	MFC average	>16	>16	>16	0.84	1.14
C. tropicalis **(n=12)**	MIC range	0.062–0.5	0.031–0.25	0.031–0.5	0.031–0.062	≤0.008
	MIC average	0.21	0.11	0.1	0.047	≤0.008
	MIC_{50}	0.25	0.125	0.031	0.031	≤0.008
	MIC_{90}	0.25	0.25	0.25	0.062	≤0.008
	MFC range	>16	>16	>16	0.25–1	0.008->4
	MFC average	>16	>16	>16	0.46	0.17
C. parapsilosis **(n=15)**	MIC range	0.062–0.5	0.031–0.25	0.031–0.25	0.062–1	0.25–2
	MIC average	0.17	0.09	0.06	0.27	1.08
	MIC_{50}	0.125	0.062	0.031	0.25	0.5
	MIC_{90}	0.25	0.062	0.062	0.5	2
	MFC range	>16	>16	1->16	1->16	2->4
	MFC average	>16	>16	3.6	3.11	2.16
C. glabrata[d] **(n=13)**	MIC range	0.031–0.25	0.031–0.062	0.031–0.25	0.031–0.062	≤0.008
	MIC average	0.12	0.04	0.07	0.05	≤0.008
	MIC_{50}	0.125	0.031	0.062	0.031	≤0.008
	MIC_{90}	0.25	0.062	0.125	0.062	≤0.008
	MFC range	>16	>16	>16	0.5–4	0.008–2
	MFC average	>16	>16	>16	1.3	0.54
C. krusei[d] **(n=10)**	MIC range	0.062–0.125	0.031–0.062	0.031–0.5	0.031–0.125	≤0.008–1
	MIC average	0.1	0.04	0.25	0.16	0.32
	MIC_{50}	0.125	0.031	0.125	0.125	0.25
	MIC_{90}	0.125	0.062	0.5	0.25	0.25
	MFC range	>16	>16	>16	1–2	0.125–2
	MFC average	>16	>16	>16	1.5	0.49
C. guilliermondii[d] **(n=3)**	MIC range	0.12–0.25	0.031–0.12	0.031–0.125	0.125–0.5	0.5–1
	MIC average	0.21	0.06	0.072	0.375	0.83
	MFC range	>16	>16	>16	>16	>16
	MFC average	>16	>16	>16	>16	>4

VRC, voriconazole; CAS, caspofingin; MFG, micafungin; MIC, the lowest concentration that inhibits 50% of fungal growth; MFC, the lowest concentration that reduces ~99.9% of fungal viability; MIC_{50}, concentration that inhibits 50% of the strains (n>10); MIC_{90}, concentration that inhibits 90% of the strains (n>10).
[a]No breakpoint values to *C. glabrata* and *C. guilliermondii*.
[b]*C. albicans* (IAL-40 and IAL-56), *C. tropicalis* (IAL-8) and *C. parapsilosis* (IAL-18) were classified as Intermediary (I) to VRC.
[c]*C. krusei* IAL-30 was resistant to MFG.
[d]Alternative taxonomic names: *Candida glabrata* (*Nakaseomyces glabrata*), *Candida guilliermondii* (*Meyerozyma guilliermondii*) and *Candida krusei* (*Pichia kudriavzerii*).

3.2. 2-aryloxazolines inhibit the proteinase activity and the total biomass formation of Candida albicans and Candida tropicalis biofilms

The proteinase activity of *C. albicans* SC5314 was significantly reduced after treatment with higher concentrations of **4i** or **9i** (32 and 64 μg/mL) when compared to the untreated cells, but no inhibitory effect was observed in *C. tropicalis* strain tested in this work (Table 3).

The 2-aryloxazolines **4i** and **9i** did not inhibit the metabolic activity of sessile cells after treatment of *C. albicans* and *C. tropicalis* biofilms, during their formation, for up to 24 h of incubation time, even at the higher concentrations of compounds (128 μg/mL, data not shown). However, when we observed the total biomass data at different incubation periods, the compound **4i** inhibited the total biomass during early biofilm formation (Fig. 2). It was more prominent against *C. albicans* where ~50% inhibition was observed, at 64–128 μg/mL for up to 12 h of biofilm formation, while ≤ 17% inhibition of total biomass was observed after 24 h. In *C. tropicalis* biofilm, there was ~35–50% inhibition of biofilm formation at concentrations of 32–128 μg/mL, in the first 4 h; after 24 h the inhibition was only 10–25% (Fig. 2).

3.3. 2-aryloxazoline derivatives inhibit the morphogenesis of Candida albicans and Candida tropicalis in different filament-inducing media

The fungal growth inhibitory effect was also associated with significant cell cycle arrest in budding yeast after treatment with **4i** or **9i** (Table 4). In addition, we observed that both compounds in RPMI medium were able to partially inhibit the filamentation in *C. albicans* and completely in *C. tropicalis* (Figs. 3, 4A-B, and 5A-B) and the inhibition of yeast-hyphae transition was considered concentration-dependent (Table S4). Therefore, as the RPMI medium induces multiple pathways for true hyphae formation, it was not possible to identify which specific filament signaling pathway was affected by the compounds.

In this regard, **4i** and **9i** were tested against *C. albicans* and *C. tropicalis* strains in other filament-inducing media as Spider broth, Lee, and SM-GlcNAc to elucidate the signaling pathway affected by the compounds. In these media, both compounds inhibited fungal growth but had MIC values higher than those found in the RPMI medium due to the nutrient rich composition (Table 5).

In addition, *C. albicans* and *C. tropicalis* cells treated with MIC values in the filament-inducing media were observed in light and fluorescence microscope. In the Lee medium (induces Cph2 and Tec1 pathways), 2-aryloxazolines partially inhibited the morphogenesis of *C. albicans*, that is, there was still the presence of shorter and less branched hyphae (Fig. 4C-D); while the compounds inhibited *C. tropicalis* filamentation and induced an enlargement of yeast size after treatment (Fig. 5C-D). Finally, it is possible to notice other two findings: i) there was no inhibition of filamentation when yeasts were treated in SM-GlcNAc that activates Ngt1 signal (Figs. 4G-H and 5G-H) and ii) the yeast-hyphae transition was totally inhibited when both species were treated in Spider broth that induces Efg1 pathway, highlighting for great alteration of yeast morphology of *C. albicans* such as reduction of cell size and induction of multiple budding/aggregates (Figs. 4E-F and 5E-F).

Table 3
Proteinase activity (Pz values) of *Candida* spp. treated with **4i** and **9i**.

Strains		Untreated	16 μg/mL	32 μg/mL	64 μg/mL
C. albicans SC 5314	**4i**	0.29 ±0.014	0.28 ±0.007	0.34 ±0.014*	0.36 ±0.007*
	9i	0.29 ±0.014	0.35 ±0.007	0.35 ±0.021*	0.37 ±0.007*
C. tropicalis IAL-01	**4i**	0.22 ±0.014	0.21 ±0.007	0.20 ±0.014	0.22 ±0.014
	9i	0.22 ±0.014	0.20 ±0.014	0.21±0.07	0.20 ±0.014

Pz: ratio between the diameter of the degradation zone around the colony and the diameter of the colony.
*$p<0.05$ when compared to untreated cells (*One way* ANOVA followed by Dunnett's test).

3.4. 2-aryloxazolines reduce virulence genes expression in Candida albicans

After treatment with **4i** at inhibitory concentration, *C. albicans* cells were analyzed for the expression of virulence genes that are expressed during transition from yeast to hyphae/pseudohyphae, such as adhesins (*HWP1* and *ALS3*), aspartyl proteinases (*SAP1* and *SAP6*), candidalysin toxin (*ECE1*), transcriptional regulators for filament induction (*CPH1, TEC1* and *EFG1*), and proteins related to the signaling pathways (*RIM8*, *CEK1*, *RAS1, CPH2, NGT1,* and *CYR1*).

Several genes were downregulated after yeast treatment with compound **4i**, but we highlighted the downregulation of adhesins (*HWP1* and *ALS3*), candidalysin *ECE1*, MAP kinase *CEK1* (1.5-fold change), and transcription factors *TEC1* (4-fold change) and *EFG1* (1-fold change) (Fig. 6). Tec1 is associated to the induction of *C. albicans* filament by the Chp2 and Efg1 pathways (Subdery, 2011). Here, we observed downregulation of *EFG1* when *C. albicans* was treated with compound in RPMI medium (induces multiple pathways) that corroborated with the partial inhibition of morphogenesis in Lee medium that induces Cph2 and Tec1 pathways and total inhibition in Spider broth that induces only Efg1 pathway. Therefore, our data suggest that inhibition of morphogenesis in *C. albicans* by 2-aryloxazoline derivatives is associated mainly with downregulation of Efg1 and Cek1 pathways.

3.5. 2-aryloxazolines induce ultrastructural alterations of Candida albicans

The untreated cells had an electron-dense and homogeneous cytoplasm content, a preserved and compacted cell wall, and an intact plasma membrane (Fig. 7A). However, 2-aryloxazoline **4i** led to some cellular effects on *C. albicans* treated with **4i** such as the loss of structural integrity of the plasma membrane, presence of electron-lucent vacuoles and low electron-density of the cell wall (Fig. 7B-C).

4. Discussion

The compounds **4i** and **9i** inhibited the fungal growth of 69 clinical and standard strains of *Candida* spp. confirming previously obtained data of antifungal activity screening of 2-aryloxazoline derivatives (Argomedo et al., 2020). Importantly, these compounds also inhibited the strains resistant to standard antifungals in addition to no positive correlation between MIC values suggesting low cross-resistance. In addition to inhibiting *Candida* growth the compounds also impaired the yeast-hyphae transition of competent-filament species *C. albicans* and *C. tropicalis* hindering the biofilm formation and proteinase activity.

The morphogenesis of some *Candida* species, i.e., transition from yeast to hyphae/pseudohyphae, is a multifactorial process influenced by nutrient composition, temperature, oxygen tension, amount of CO_2, pH, small molecules, and extracellular matrix (Sudbery, 2011; Vila et al., 2017). *C. albicans* uses a complex cell morphology control circuit, that the number of positive regulators of morphogenesis that allow filamentation in response to signals greatly exceeds the number of negative regulators that maintain the yeast growth form in the absence of inducers (O'meara et al., 2015). The morphogenesis regulation is carried out by signals and transduction factors, which can induce hypha formation in several ways by the positive regulators (Efg1, Cph1, Cph2, Tec1, Flo8, Czf1, Rim101, and Ndt80) or inhibit by the negative regulators (Tup1, Nrg1, Rox1p, and Rfg1) (Sudbery, 2011). During this cellular event in *C. albicans* and *C. tropicalis* other genes are also down- and upregulated reflecting not only on shape change but on metabolism, expression of virulence factors and tolerance mechanisms (Lane et al., 2001; Naglik et al., 2003; Schweizer et al., 2000, Sudbery, 2011, Zuza-Alves et al., 2017; Santos and Ishida, 2023).

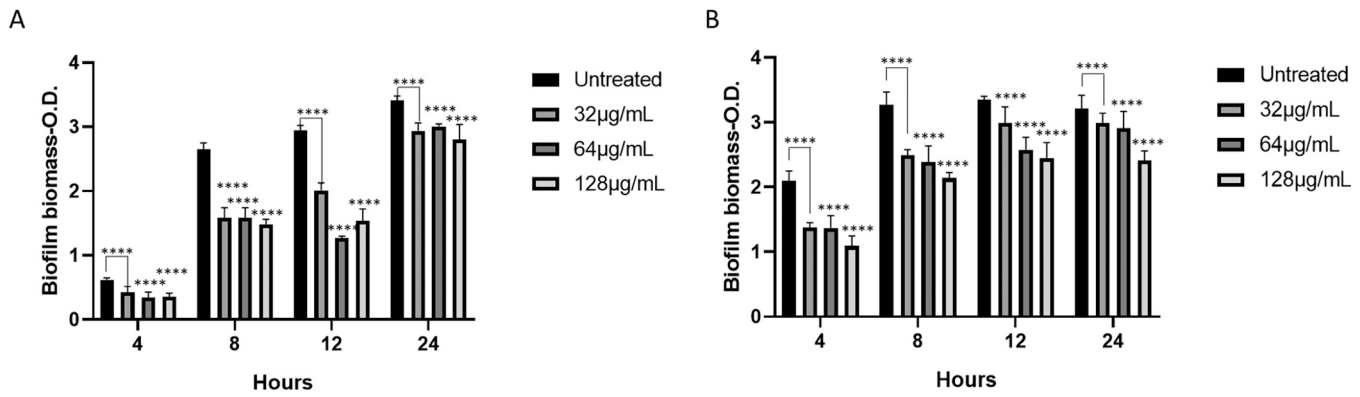

Fig. 2. Measurement of total biomass during biofilm formation of *Candida albicans* SC5314 and *Candida tropicalis* IAL-01 at different incubation times of untreated and treated with the 2-aryloxazoline **4i**. (A) Total biomass of *C. albicans* biofilm and (B) total biomass of *C. tropicalis* biofilm after staining with crystal violet. ****p<0.0001 when compared to untreated biofilms (*One way* ANOVA followed by Dunnett's test).

Table 4
Budding percentage of *Candida* spp. strains treated with MIC values of 2-aryloxazoline derivatives **4i** and 9i.

Strains	Budding percentage (%)		
	Untreated	**4i**	**9i**
C. albicans SC5314	41.17±2.22	68.30±1.18*	61.68±3.91*
C. tropicalis IAL-01	13.01±1.26	22.81±0.007*	53.74±7.12*

* p<0.05 when compared with untreated yeasts (*One way* ANOVA followed by Dunnett's test).

The morphogenesis inhibition in *C. albicans* or *C. tropicalis* by 2-aryloxazolines was observed after microdilution assay using RPMI, Spider, and Lee media. The yeast-hyphae transition was completely inhibited by the compounds in Spider broth (actives only Efg1 pathway) and partially inhibited in Lee medium (induces Cph2 and Tec1 pathways) and RPMI medium (actives multiple pathways). Therefore, the results obtained of filament-inducing media associated to expression data of the virulence genes suggested that 2-aryloxazolines inhibit the morphogenesis by hinder the cyclic AMP-dependent pathway (cAMP) that activates the enhanced filamentous growth protein 1 (Efg1), a transcription factor key in the hyphae development. This observation can be supported by downregulation of *EFG1* and *TEC1* genes in the 4i-treated cells. The gene expression of *TEC1* is also regulated by *CPH2* in addition to the *EFG1* gene (Subdery, 2011). *CPH2* gene had no change in its expression in ratio to the untreated and treated cells, indicating that downregulated *TEC1* was not associated with Cph2 pathway corroborating with morphology results obtained in the Lee medium.

Efg1 can also be activated by different ways, e.g., cAMP (Cyr1-Ras1), Rim101, and Ngt1, for induction of hyphae formation filament formation (Subdery, 2011). Here, we also observed a slight downregulation of

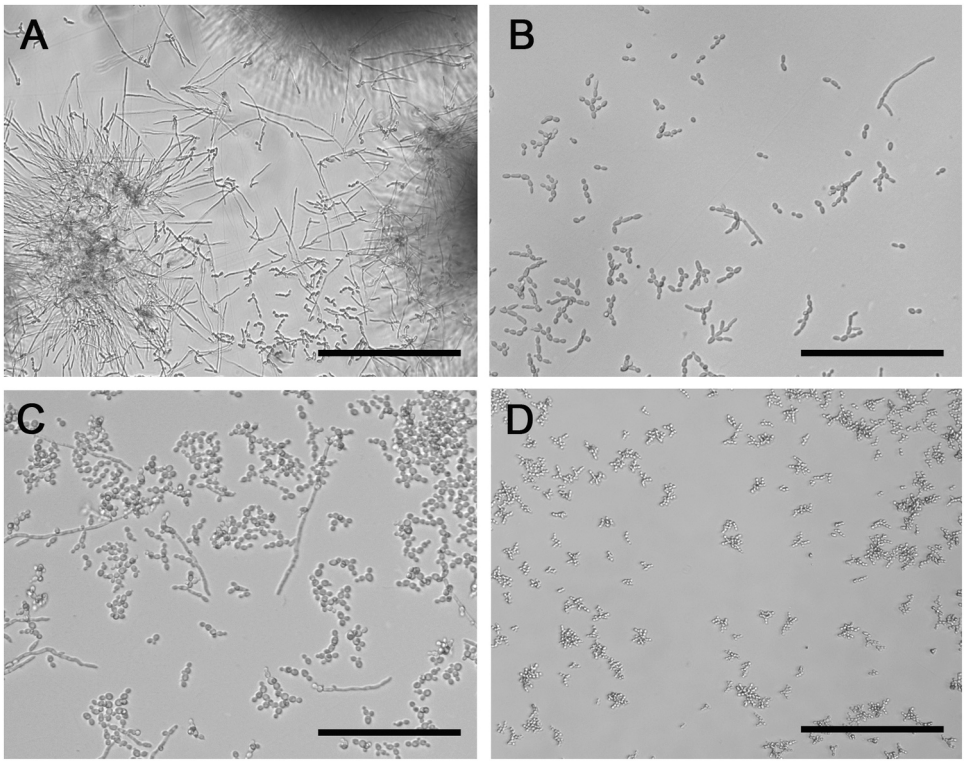

Fig. 3. *Candida albicans* SC5314 (A-B) and *Candida tropicalis* IAL-01 (C, D) visualized by light microscopy. (A, C) untreated cells. (B, D) **4i**-treated cells. Similar results were observed when cells were treated with **9i**. Bars = 100 µm.

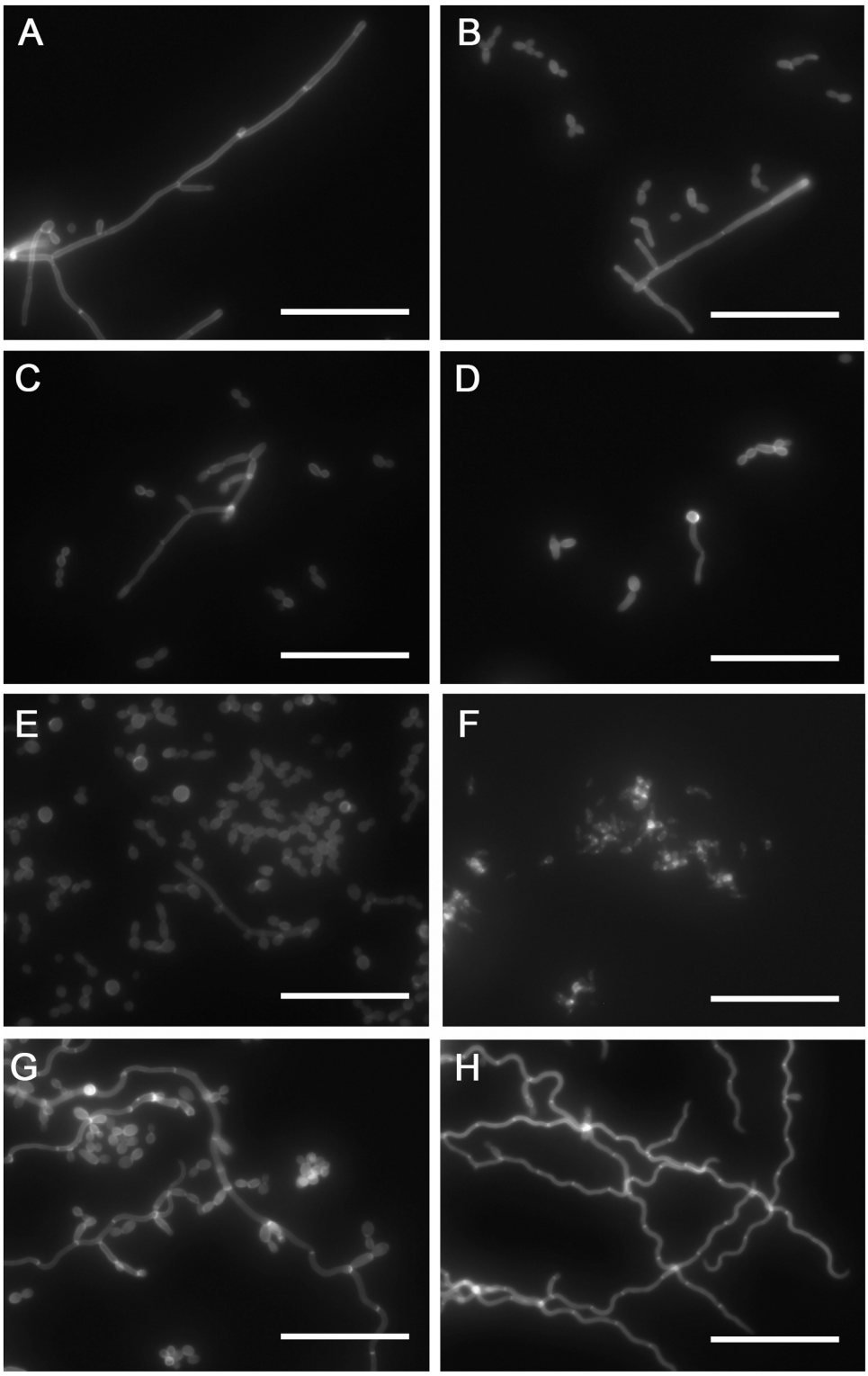

Fig. 4. Fluorescence microscopy images of *Candida albicans* SC5314 untreated (left) and treated with **4i** (right) in filament-inducer liquid media and stained with calcofluor white. (A, B) RPMI, (C, D) Lee, (E, F), Spider, and (G, H) SM medium supplemented with N-acetylglucosamine. Similar results were observed when cells were treated with **9i**. Bars = 50 μm.

CYR1 and *RIM8* genes that are involved in the Efg1 pathway. On the other hand, no inhibition of the yeast-hyphae transition was observed in the 4i-treated cells in SM-GlcNAc medium. This medium contains N-acetylglucosamine as primary carbon source that is transported to cytoplasm of *C. albicans* and *C. tropicalis* using the transmembrane protein Ngt1 (Alvarez & Konopka, 2007; Gilmore et al., 2013; Zhang et al., 2020). Besides, our data of *NGT1* gene expression did not show a relevant change in cells treated with 4i suggesting that the compound did not inhibit morphogenesis by this way.

The RPMI medium is a chemically defined medium and includes multiple factors and components essential for hyphal induction (O'meara et al., 2015; Sato, Hoshida, Akada, 2020). The treatment of

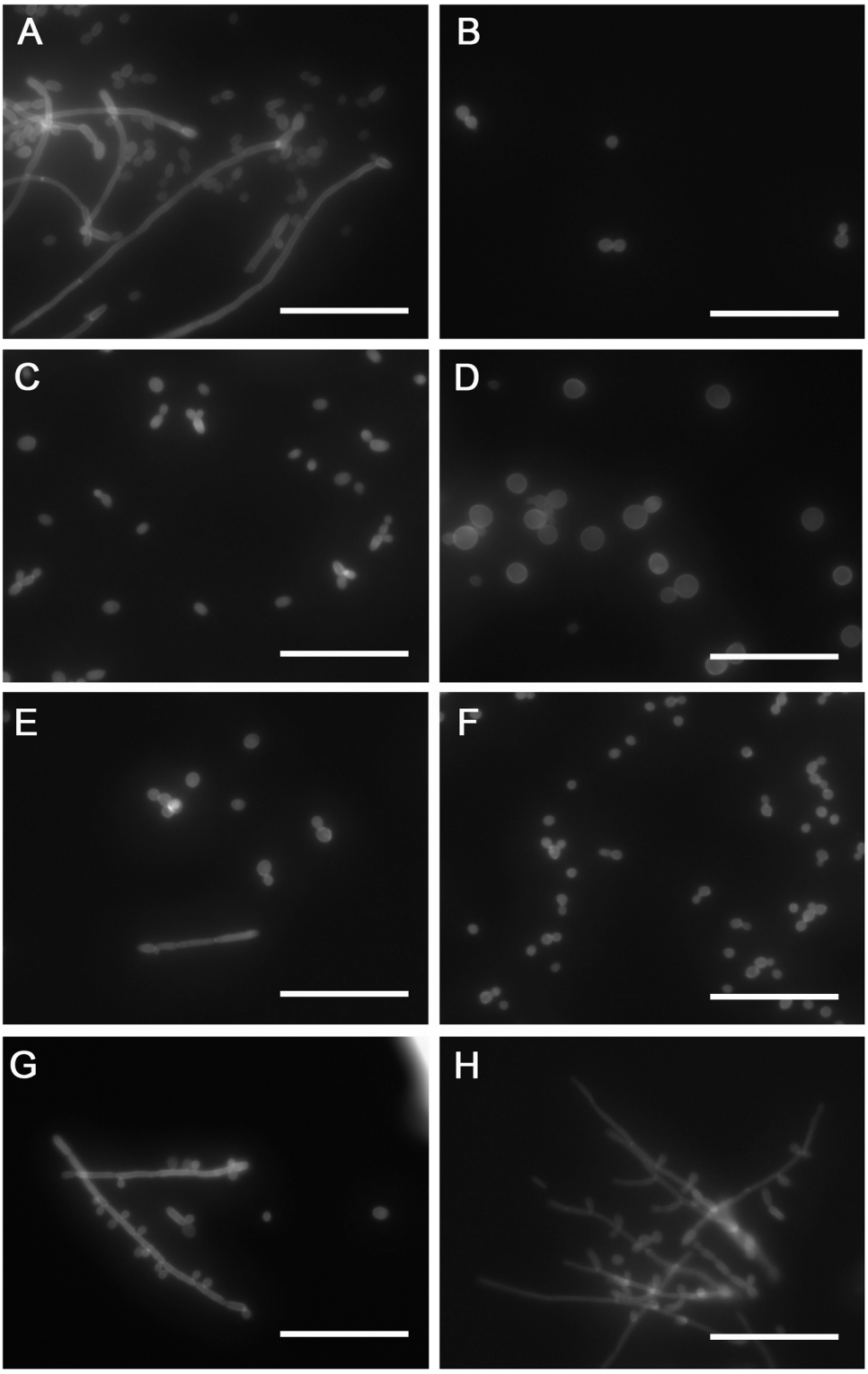

Fig. 5. Fluorescence microscopy images of *Candida tropicalis* IAL-01 untreated (left) and treated with **4i** (right) in filament-inducer liquid media and stained with calcofluor white. (A, B) RPMI, (C, D) Lee, (E, F), Spider, and (G, H) SM medium supplemented with N-acetylglucosamine. Similar results were observed when cells were treated with **9i**. Bars = 50 μm.

C. albicans with compounds, at MIC values, in RPMI medium did not suppress the total formation of hyphae, in which a population mixture of yeast, pseudohyphae, and hyphae was observed. In fact, several complex networks of the morphogenesis signaling pathways are being activated beyond cAMP-dependent pathways, e.g., MAPK (mitogen-activated protein kinase) pathways (Xie et al., 2016). Four MAPK pathways have been identified in *C. albicans*: Hog pathway, the cell wall integrity Mkc1 pathway, and Cek1 and Cek2 pathways. The MAPK pathways are involved in the adaptation to both osmotic and oxidative stresses, biogenesis and repair of the cell wall, hyphal development, biofilm formation, and virulence (Csank et al., 1998; Enjalbert et al., 2005; Li et al., 2015; Navarro-Garcia et al., 2015). In this work, we also observed

Table 5
Inhibitory effect of compounds **4i** and **9i** on *Candida albicans* and *Candida tropicalis* in different hyphae-inducing media. The MIC data are expressed in μg/mL.

Strains	RPMI		Spider		Lee		SM-GlcNAc	
	4i	9i	4i	9i	4i	9i	4i	9i
C. albicans SC 5314	0.06	0.03	8	16	1	1	1	4
C. albicans IAL-40	0.12	0.03	8	8	2	2	0.5	4
C. albicans ATCC 10231	0.06	0.03	8	4	2	2	2	2
C. tropicalis IAL-01	2	4	2	4	4	4	2	2
C. tropicalis IAL-04	0.25	0.25	16	16	2	2	0.03	0.5

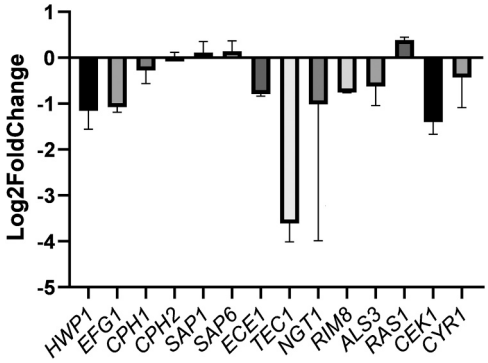

Fig. 6. Quantification of virulence genes expression from *Candida albicans* SC5314 cells treated with compound **4i**, at inhibitory concentration, in relation to untreated cells in RPMI medium for 24 h at 35 °C. Reverse transcription quantitative PCR (RT-qPCR) was performed to quantify the expression levels of genes during *C. albicans* filamentation. Data were normalized to the reference gene beta-actin. The assay was performed in duplicate.

that *CEK1* gene was downregulated in **4i**-treated cells. The Cek1 (*Candida* ERK-like kinase) pathway is implicated in cell wall biogenesis, hyphal development, biofilm formation, and virulence as previously described (Csank et al., 1998; Enjalbert et al., 2005; Li et al., 2015; Navarro-Garcia et al., 2005) corroborating with inhibition of filamentation and biofilm formation as well as alterations in the fungal cell wall ultrastructure after **4i**-treatment. Then, the *CEK1* gene expression was 1.5-fold change lower suggesting that 2-aryloxazoline derivatives also inhibit the morphogenesis by *CEK1* downregulation.

Taken together, 2-aryloxazoline derivatives were able to inhibit yeast-hyphae transition of *C. albicans* mainly by downregulation of Efg1 and Cek1 pathways. In this way, consequently, other virulence genes related with morphogenesis were also affected such as the adhesins (*HWP1* and *ALS3)* and candidalysin toxin (*ECE1*) genes that their expression were downregulated ~2-fold-change after **4i**-treatment.

The Hyphal wall protein (Hwp1) is a well-characterized *C. albicans* cell wall surface protein, through different mechanisms, enable the fungus to adhere tightly to host tissues, e.g., oral, gastrointestinal, and vaginal mucosa, and therefore it is considered essential for *Candida* spreading and invasiveness (Naglik et al., 2006; Nobile and Mitchell., 2006; Maras et al., 2021). The agglutinin-like protein 3 (Als3) is a cell surface adhesion protein expressed in hyphae that plays an important role in the biofilm formation of *C. albicans* and tissue invasion and dissemination (Liu et al., 2021). Both adhesins were downregulated in the treatment with 2-aryloxazoline, suggesting that this compound interfered with this mechanism of the virulence. In addition, the fungal peptide toxin candidalysin is derived from a larger parental pre-proprotein (Ece1p) encoded by *ECE1* gene expressed during hyphal growth (Richardson et al., 2018). Candidalysin has cytolytic and immunostimulatory actions leading to tissue damage; it promotes the influx of cytokines into the epithelium and represents a critical virulent factor for mucosa and systemic infection of *C. albicans* (Naglik et al., 2019; Macias-Paz et al., 2023).

5. Conclusion

The 2-aryloxazoline compounds inhibited the growth of *Candida* spp. clinical strains, including isolates resistant and less susceptible to standard antifungals. Although the compounds did not inhibit the metabolic activity of cells from biofilms, in formation, they reduced the total biomass in the early stages of biofilm formation of *C. albicans* and *C. tropicalis*. The inhibition of yeast-hyphae transition in *C. albicans* and *C. tropicalis* using different filamentation-inducing media and the gene expression results suggest that the compounds downregulated some genes associated with the filamentation-inducing pathways cAMP and MAPK. Future studies are needed to elucidate the other mechanisms of action for antifungal activity showed by the 2-aryloxazoline compounds on *Candida* species.

Ethical Approval

Not required.

Funding

This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, 2021/01279–5 and 2020/00295–4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 405556/2018–7) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). VMB was FAPESP fellow (2018/11612–0). KI and HAS are research fellows of the CNPQ (306041/2022–7 and

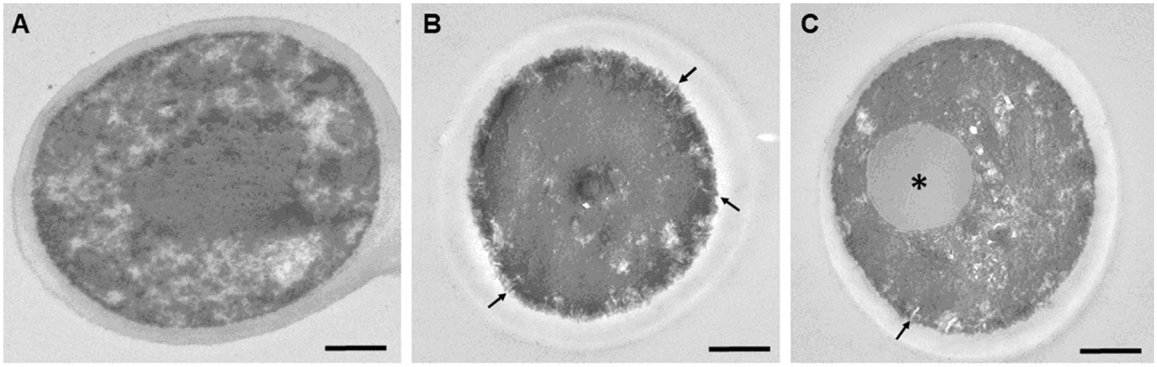

Fig. 7. Ultrastructural effects of 2-aryloxazoline compound on *Candida albicans* SC5314. (A) Untreated cell and (B and C) cells treated with inhibitory concentration of **4i**. The compound led to some cellular changes, such as the accumulation of electron-lucent vacuoles (asterisk), loss of the plasma membrane integrity (arrows), and low electron density of cell wall. Bars = 500 nm.

311761/2022–4, respectively).

CRediT authorship contribution statement

Kelly Ishida: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hélio A. Stefani:** Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Joel S. Reis:** Methodology, Investigation, Formal analysis, Data curation. **Vinícius M. Barroso:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Kelly Ishida reports financial support was provided by State of Sao Paulo Research Foundation. Kelly Ishida has patent #BR 10 2017 023661 7 issued to Licensee. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank Prof. Dr. Benedito Corrêa for kindly sharing the framework for molecular biology studies and Dr. Tatiana Reis and Marcela Gonçalves for technical support in the laboratory.

Author contributions

VMB performed the experiments, analyzed the results, and wrote the manuscript. HAS and JSR were responsible for the synthesis of 2-aryloxazolines. KI designed the experiments, analyzed the data, wrote, and edited the manuscript. All authors have read and approved the manuscript before publication.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2024.100062.

References

Alonso, G.C., Pavarina, A.C., Sousa, T.V., Klein, M.I., 2018. A quest to find good primers for gene expression analysis of *Candida albicans* from clinical samples. J. Microbiol. Methods 147, 1–13. https://doi.org/10.1016/j.mimet.2018.02.010.

Alvarez, F.J., Konopka, J.B., 2007. Identification of an N-acetylglucosamine transporter that mediates hyphal induction in *Candida albicans*. Mol. Biol. Cell.,18 965–975. https://doi.org/10.1091/mbc.E06-10-0931.

Alves, C.T., Wei, X.-Q., Silva, S., Azeredo, J., Henriques, M., Williams, D.W., 2014. *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. J. Infect. 69 (4), 396–407. https://doi.org/10.1016/j.jinf.2014.06.002.

Arendrup, M.C., Patterson, T.F., 2017. Multidrug-Resistant *Candida*: Epidemiology, Molecular Mechanisms, and Treatment. J. Infect. Dis. 216 (3), 445–451. https://doi.org/10.1093/infdis/jix131.

Argomedo, L.M.Z., Barroso, V.M., Barreiro, C.S., Darbem, M.P., Ishida, K., Stefani, H.A., 2020. A. Novel 2 Aryloxazoline Compounds exhibit an inhibitory effect on *Candida* spp., including antifungal-resistant isolates. ACS Med. Chem. Lett. 11, 2470–2475. https://doi.org/10.1021/acsmedchemlett.0c00449.

Bongomin, F., Gago, S., Oladele, R.O., Denning, D.W., 2017. Global and Multi-national Prevalence Of Fungal Diseases—estimate Precision. J. Fungi 3 (57), 1–29. https://doi.org/10.3390/jof3040057.

Castilla, R., Passeron, S., Cantore, M.L., 1998. N-acetyl-D-glucosamine induces germination in *Candida albicans* through a mechanism sensitive to inhibitors of cAMP-dependent protein kinase. Cell Signal 10, 713–719. https://doi.org/10.1016/s0898-6568(98)00015-1.

Csank, C., Schröppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., Thomas, D. Y., Whiteway, M., 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect. Immun. 66 (6), 2713–2721. https://doi.org/10.1128/IAI.66.6.2713-2721.1998.

Clinical Laboratory Standard Institute (CLSI). 2017. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. 4th ed. CLSI standard M27. Wayne, PA: Clinical and Laboratory Standards Institute.

Clinical Laboratory Standard Institute (CLSI). 2022. Performance Standards for Antifungal Susceptibility Testing of Yeasts. 3rd ed. CLSI supplement M27M44S. Clinical and Laboratory Standards Institute.

Enjalbert, B., Smith, D.A., Cornell, M.J., Alam, I., Nicholls, S., Brown, A.J.P., Quinn, J., 2005. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. Mol. Biol. Cell. 17 (2), 1018–1032. https://doi.org/10.1091/mbc.e05-06-0501.

Freitas, A.L.D., Kaplum, V., Rossi, D.C.P., Da Silva, L.B., Melhem, M.S.C., Taborda, C.P., Mello, J.C.P., Nakamura, C.V., Ishida, K., 2018. Proanthocyanidin polymeric tannins from *Stryphnodendron adstringens* are effective against *Candida* spp. isolates and for vaginal candidiasis treatment. J. Ethnopharmacol. 216, 184–190. https://doi.org/10.1016/j.jep.2018.01.008.

Gilmore, S.A., Naseem, S., Konopka, J.B., Sil, A., 2013. N-acetylglucosamine (GlcNAc) triggers a rapid, temperature-responsive morphogenetic program in thermally dimorphic fungi. PLoS Genet 9 (9). https://doi.org/10.1371/journal.pgen.1003799.

Kalimuthu, S., Alshanta, O.A., Brishnamoorthy, A.L., Pudipeddi, A., Solomon, A.P., McLean, W., Leung, Y.Y., Ramage, G., Neelakantan, P., 2022. Small molecule based anti-virulence approaches against *Candida albicans* infections. Crit. Rev. Microbiol. 48 (6), 743–769. https://doi.org/10.1080/1040841X.2021.2025337.

Lane, S., Birse, C., Zhou, S., Matson, R., Liu, H., 2001. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. J. Biol. Chem. 276 (52), 48988–48996. https://doi.org/10.1074/jbc.M104484200.

Lass-Florl, C., Kanj, S.S., Govender, N.P., Thompson III, G.R., Ostrosky-Zeichner, L., Govrins, M.A., 2024. Invasive candidiasis. Nat. Rev. Dis. Prim. 10 (20), 1–18. https://doi.org/10.1038/s41572-024-00503-3.

Li, R., Puri, S., Tati, S., Cullen, P.J., Edgerton, M., 2015. *Candida albicans* Cek1 Mitogen-Activated Protein Kinase Signaling Enhances Fungicidal Activity of Salivary Histatin 5. Antimicrob. Agents Chemother. 59 (6), 3460–3468. https://doi.org/10.1128/AAC.00214-15.

Liu, C., Xu, C., Du, Y., Liu, J., Ning, Y., 2021. Role of agglutinin-like sequence protein 3 (Als3) in the structure and antifungal resistance of *Candida albicans* biofilms. FEMS Microbiol. Lett. 368 (14), fnab089. https://doi.org/10.1093/femsle/fnab089.

Macias-Paz, I.U., Pérez-Hernándes, S., Tavera-Tapia, A., Luna-Arias, J.P., Guerra-Cárdenas, J.E., Reyna-Beltrán, E., 2023. *Candida albicans* the main opportunistic pathogenic fungus in humans. Ver. Argent. Microbiol. 55 (2), 189–198. https://doi.org/10.1016/j.ram.2022.08.003.

Maras, B., Maggiore, A., Mignogna, G., D'Erme, M., Angiolella, L., 2021. Hyperexpression of *CDRs* and *HWP1* genes negatively impacts on *Candida albicans* virulence. PloS One 16 (6). https://doi.org/10.1371/journal.pone.0252555.

Mardegan, R.C., Klein, M.I., Golvea, M.B., Rodrigues, J.A.O., Gonçalves, R.B., Höfling, J. F., 2006. Biotyping and genotypic diversity among oral *Candida albicans* strains from caries-free and caries-active healthy children. Braz. J. Microbiol. 37 (1), 26–32. https://doi.org/10.1590/S1517-83822006000100005.

Mukherjee, P.K., Sheehan, D.J., Hitchcock, C.A., Ghannoum, M.A., 2005. Combination treatment of invasive fungal infections. Clin. Microbiol. Rev. 18 (1), 163–194. https://doi.org/10.1128/CMR.18.1.163-194.2005.

Muñoz, J.E., Rossi, D.C.P., Ishida, K., Spadari, C.C., Melhem, M.S.C., Garcia, D.M., Caires, A.C.F., Taborda, C.P., Rodrigues, E.G., 2017. Antifungal activity of the biphosphinic cyclopalladate C7a against *Candida albicans* yeast forms *in vitro* and *in vivo*. Front. Microbiol. 8, 1–10. https://doi.org/10.3389/fmicb.2017.00771.

Murphy, S.E., Bicanic, T., 2021. Drug Resistance and Novel Therapeutic Approaches in Invasive Candidiasis. Front. Cell Infect. Microbiol. 11, 1–22. https://doi.org/10.3389/fcimb.2021.759408.

Naglik, J.R., Challacombe, S.J., Hube, B., 2003. *Candida albicans* Secreted Aspartyl Proteinases in Virulence and Pathogenesis. Microbiol. Mol. Biol. Rev. 67 (3), 400–428. https://doi.org/10.1128/MMBR.67.3.400-428.2003.

Naglik, J.R., Fostira, F., Ruprai, J., Staab, J., Challacombe, S.J., Sundstrom, P., 2006. *Candida albicans HWP1* gene expression and host antibody responses in colonization and disease. J. Med. Microbiol. 55 (10), 1323–1327. https://doi.org/10.1099/jmm.0.46737-0.

Naglik, J.R., Gaffen, S.L., Hube, B., 2019. Candidalysin: discovery and function in *Candida albicans* infections. Curr. Opin. Microbiol. 52, 100–109. https://doi.org/10.1016/j.mib.2019.06.002.

Navarro-Garcia, F., Eisman, B., Fiuza, S.M., Nombela, C., Pla, J., 2005. The MAP kinase Mkc1p is activated under different tress conditions in *Candida albicans*. Microbiol 151 (8), 2737–2749. https://doi.org/10.1099/mic.0.28038-0.

Nobile, J., Mitchell, A.P., 2006. Genetics and genomics of *Candida albicans* biofilm formation. Cell. Microbiol. 8 (9), 1382–1391. https://doi.org/10.1111/j.1462-5822.2006.00761.x.

O'meara, T.R., Veri, A.O., Ketela, T., Jiang, B., Roemer, T., Cowen, L.E., 2015. Global analysis of fungal morphology exposes mechanisms of host cell escape. Nat. Commun. 6, 6741. https://doi.org/10.1038/ncomms7741.

Pappas, P.G., Kauffman, C.A., Andes, D.R., Clancy, C.J., Marr, K.A., Ostrosky-Zeichner, L., Reboli, A.C., Schuster, M.G., Vasquez, J., Walsh, T.J., Zaoutis, T.E., Sobel, J.D., 2016. Clinical Practice Guideline for the Management of Candidiasis:

2016 Update by the Infectious Diseases Society of America. Clin. Infect. Dis. 62 (4), e1–e50. https://doi.org/10.1093/cid/civ933.
Perlin, D.S., Rautemaa-Richardson, R., Alastruey-Izquierdo, A., 2017. The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect. Dis. 17 (12), 383–392. https://doi.org/10.1016/S1473-3099(17)30316-X.
Pierce, C.G., Chaturvedi, A.K., Lazzell, A., Powell, A.T., Saville, S.P., McHardy, S.F., Lopez-Ribot, J.L., 2015. A novel small molecule inhibitor of *Candida albicans* biofilm formation, filamentation and virulence with low potential for the development of resistance. NPJ Biofilms Micro 1, 15012. https://doi.org/10.1038/npjbiofilms.2015.12.
Ramos, L.S., Figueiredo-Carvalho, M.H.G., Barbedo, L.S., Ziccardi, M., Chaves, A.L.S., Zancopé-Oliveira, R.M., Pinto, M.R., Sgarbi, D.B.G., Dornelas-Ribeiro, M., Branquinha, M.H., Santos, A.L.S., 2015. *Candida haemulonii* complex: species identification and antifungal susceptibility profiles of clinical isolates from Brazil. J. Antimicrob. Chemother. 70 (1), 111–115. https://doi.org/10.1093/jac/dku321.
Richardson, J.P., Mogavero, S., Moyses, D.L., Blagojevic, M., Krüger, T., Verma, A.H., Coleman, B.M., Diaz, J.C., Schulz, D., Ponde, N.O., Carrano, G., Kniemeyer, O., Wilson, D., Bader, O., Enoiu, S.I., Ho, J., Kichik, N., Gaffen, S.L., Hube, B., Naglik, J. R., 2018. Processing of *Candida albicans* Ece1p is critical for candidalysin maturation and fungal virulence. e02178-17 mBio 9, 23. https://doi.org/10.1128/mBio.02178-17.
Rodriguez, L., Bustamante, B., Huaroto, L., Agurto, C., Illescas, R., Ramirez, R., Diaz, A., Hidalgo, J., 2017. A multi-centric Study of *Candida* bloodstream infection in Lima-Callao, Peru: species distribution, antifungal resistance and clinical outcomes. PLoS One 12 (4), e0175172. https://doi.org/10.1371/journal.pone.0175172.
Romo, J.A., Pierce, C.G., Chaturvedi, A.K., Lazzell, A.L., McHardy, S.F., Saville, S.P., Lopez-Ribot, J.L., et al., 2017. Development of Anti-Virulence Approaches for Candidiasis via a Novel Series of Small-Molecule Inhibitors of *Candida albicans* Filamentation. e01991-17 mBio 8 (6). https://doi.org/10.1128/mBio.01991-17.
Salci, T.P., Negri, M., Abadio, A.K.R., Svidzinski, T.I.E., Kioshima, E.S., 2018. Targeting *Candida* spp. to develop antifungal agents. Drug Discov. Today 23 (4), 802–814. https://doi.org/10.1016/j.drudis.2018.01.003.
Santos, M.M., Ishida, K., 2023. We need to talk about Candida tropicalis: virulence factors and survival mechanisms. Med. Mycol. 61 (8), myad075. https://doi.org/10.1093/mmy/myad075.
Sato, T., Hoshida, H., Akada, R., 2020. Inhibition of Distinct Proline- or N-Acetylglucosamine-Induced Hyphal Formation Pathways by Proline Analogs in *Candida albicans*. Biomed. Res. Int. 2020, 7245782 https://doi.org/10.1155/2020/7245782.
Schweizer, A., Rupp, S., Taylor, B.N., Röllinghoff, M., Schröppel, K., 2000. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. Mol. Microbiol. 38 (3), 435–445. https://doi.org/10.1046/j.1365-2958.2000.02132.x.
Sears, D., Schwartz, B.S., 2017. *Candida auris*: An emerging multidrug-resistant pathogen. Int. J. Infect. Dis. 63, 95–98. https://doi.org/10.1016/j.ijid.2017.08.017.
Sudbery, P.E., 2011. Growth of *Candida albicans* hyphae. Nat. Rev. Microbiol 9, 737–748. https://doi.org/10.1038/nrmicro2636.
Vila, T., Romo, J.A., Pierce, C.G., McHardy, S.F., Saville, S.P., Lopez-Ribot, J.L., 2017. Targeting *Candida albicans* filamentation for antifungal drug development. Virulence 8 (2), 150–158. https://doi.org/10.1080/21505594.2016.1197444.
Xie, J.L., Grahl, N., Sless, T., Leach, M.D., Kim, S.H., Hogan, D.A., Robbins, N., Cowen, L. E., 2016. Signaling through Lrg1, Rho1 and Pkc1 Governs *Candida albicans* Morphogenesis in Response to Diverse Cues. PLOS Genet 12 (10), 1–22. https://doi.org/10.1371/journal.pgen.1006405.
Yap, H.Y., Kwok, K.M., Gomersall, C.D., Fung, S.C., Lam, T.C., Leung, P.N., Hui, M., Joynt, G.M., 2009. Epidemiology and outcome of *Candida* bloodstream infection in an intensive care unit in Hong Kong. Hong. Kong Med. J. 15, 255–261.
World Health Organization (WHO). 2022. WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization. Licence: CC BY-NC-SA 3.0 IGO.
Zhang, Q., Xu, L., Yuan, S., Zhou, Q., Whang, X., Whang, L., Hu, Z., Yan, Y., 2020. NGT1 Is Essential for N-Acetylglucosamine-Mediated Filamentous Growth Inhibition and HXK1 Functions as a Positive Regulator of Filamentous Growth in *Candida tropicalis* (https://doi:). Int J. Mol. Sci. 21 (11). https://doi.org/10.3390/ijms21114036.
Zuza-Alves, D.L., Silva-Rocha, W.P., Chaves, G.M., 2017. An Update on *Candida tropicalis* Based on Basic and Clinical Approaches. Front. Microbiol. 8 (1927), 1–25. https://doi.org/10.3389/fmicb.2017.01927.