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Beatriz Virgínia da Silva

Indução *in vitro* de resistência ao fluconazol e caspofungina em espécies do complexo *Candida parapsilosis*: implicações para virulência e resistência cruzada a outros antifúngicos

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BEATRIZ VIRGÍNIA DA SILVA

INDUÇÃO IN VITRO DE RESISTÊNCIA AO FLUCONAZOL E CASPOFUNGINA EM ESPÉCIES DO COMPLEXO CANDIDA PARAPSILOSIS: IMPLICAÇÕES PARA VIRULÊNCIA E RESISTÊNCIA CRUZADA A OUTROS ANTIFÚNGICOS

Tese apresentada ao Programa de Pós-Graduação em **CIÊNCIAS FISIOLÓGICAS**, área de concentração "**PARASITOLOGIA, IMUNOLOGIAE MICROBIOLOGIA**" (Linha de Pesquisa: **BIOLOGIA DE FUNGOS E BACTÉRIAS**) da Universidade Federal do Triângulo Mineiro como requisito parcial para obtenção do título de doutora.

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RESUMO

As espécies do gênero *Candida* frequentemente causam doenças em humanos, variando de infecções superficiais a infecções invasivas potencialmente fatais. Dentro deste gênero, *Candida albicans*, que geralmente é suscetível a antifúngicos, tem sido historicamente a espécie mais prevalente recuperada de amostras clínicas. No entanto, com o uso crescente de agentes antifúngicos e o desenvolvimento de novas técnicas de diagnóstico, está havendo uma mudança na epidemiologia das infecções por *Candida* spp. Esta mudança levou a um aumento nas infecções causadas por espécies de não *Candida albicans*, que têm uma sensibilidade antifúngica menos previsível. Entre essas espécies, o complexo *Candida parapsilosis* tem atraído atenção como agente de infecções fúngicas invasivas, particularmente prevalente entre neonatos com baixo peso ao nascer, indivíduos imunocomprometidos e pacientes que necessitam de uso prolongado de cateteres venosos centrais. O estudo teve como objetivo investigar o impacto da exposição progressiva ao fluconazol e a caspofungina em 6 *C. parapsilosis* sensu stricto, 6 *C.orthopsilosis* e 5 *C. metapsilosis*, em relação ao potencial de resistência cruzada e na estabilidade dos fenótipos de resistência após subcultura em meio livre de drogas. Além disso, avaliamos os efeitos da resistência induzida nos principais fatores de virulência, incluindo a produção de enzimas hidrolíticas e a formação de biofilme, bem como a virulência *in vivo* usando o modelo *Galleria mellonella*. A exposição progressiva ao fluconazol e à caspofungina levou ao desenvolvimento de resistência a esses antifúngicos. Notavelmente, o desenvolvimento de resistência a um agente antifúngico influenciou na sensibilidade a outros antifúngicos das classes dos azóis e equinocandinas. Apesar dessas alterações na sensibilidade antifúngica, não foram observadas alterações significativas na secreção de enzimas hidrolíticas (esterase, fosfolipase, protease e hemolisina). No entanto, a aquisição de resistência foi associada a uma maior capacidade das espécies do complexo *C. parapsilosis* em formar biofilmes, um fator importante na patogenicidade desses microrganismos. Apesar disso, a indução da alteração do perfil de sensibilidade não alterou significativamente a virulência da levedura *in vivo*, avaliada no modelo *Galleria mellonella*. Assim, este estudo fornece conhecimento significativo sobre as respostas adaptativas de espécies do complexo *C. parapsilosis* à pressão antifúngica, ressaltando a necessidade de vigilância contínua para controlar as infecções causadas por esses patógenos.

Palavras-chaves: Complexo *Candida parapsilosis*; Resistência antifúngica; Fluconazol; Capofungina; Formação de biofilme; Esterase; Fosfolipase; Proteinase; Hemolisina.

LISTA DE ABREVIATURAS E SIGLAS

AMB- Anfotericina B

ATCC - *American Type Culture Collection*

$CaCl_2$ - Cloreto de cálcio

CAS- Caspofungina

CO_2 - Dióxido de carbono

CLSI - *Clinical and Laboratory Standards Institute*

CVV- *Candida* vulvovaginal.

CNA- *Candida* não a*lbicans*

CP - *Candida parapsilosis*

FPPL- Lista de patógenos prioritários fúngicos

FLC- Fluconazol

ITS - Espaço transcrito interno

ITC - Itraconazol

K_2HPO_4 - Fosfato de potássio

MIC - Concentração Inibitória Mínima

$MgCl_2$ - Cloreto de Magnésio

$MgSO_4.7H_2O$ - Sulfato de Magnésio

mL - Mililitro

MLST- Sequenciamento em multilocus

mol/L - Molaridade

mg - Miligrama

NaCl- Cloreto de Sódio

OMS - Organização Mundial da Saúde

OD - Densidade Óptica

PBS -Tampão salina fosfatada

pH - Potencial hidrogeniônico

Pz - Zona de precipitação

R - Resistente

RPMI - *Meio Roswell Park Memorial Institute*

S - Sensível

SDD – Sensibilidade dose dependente

SDA- Ágar Sabouraud dextrose

Saps - proteinases

UFC/mL - Unidade formadora de colônias por mililitro

UTI - Unidade de Terapia Intensiva

XTT - (2,3-bis (2-metoxi-4-nitro-5-sulfofenil) -5 - [(fenilamino) carbonil] – 2Htetrazólio hidróxido)

µg - Micrograma

µL – Microlitros

µg/mL – Microgramas por mililitro

VRC - Voriconazol

SUMÁRIO

1 REVISÃO DA LITERATURA

1.1 CONSIDERAÇÕES GERAIS

Infecções causadas por fungos mostraram um substancial aumento nas últimas décadas. Esses microrganismos podem causar desde infecções superficiais, afetando a pele, cabelo, unhas e membranas mucosas, até infecções sistêmicas. Estima-se que as doenças fúngicas afetam anualmente mais de um bilhão de pessoas e causam um milhão e meio mortes no mundo (Branco et al., 2023). A gravidade das infecções é influenciada por vários fatores, como a utilização de dispositivos médicos, pacientes imunocomprometidos, crianças abaixo de um ano de idade e idosos acima de setenta anos (Mahfouz et al., 2003; Pereira et al., 2010; Brown et al., 2012; Yapar, 2014).

Dentre os agentes destas infecções fúngicas podemos citar as espécies do gênero *Candida*, sendo amplamente associadas a altas taxas de doenças graves e são responsáveis por até 30% de todas as mortes por doenças fúngicas (Strollo et al., 2016). Das espécies desse gênero, *C. albicans* tem sido historicamente relatada como a mais prevalente, embora seu predomínio tenha diminuído nos últimos anos, à medida que aumentou o número de infecções por espécies de não *Candida albicans* (CNA), como *C. parapsilosis*, *C. glabrata*, *C. tropicalis* e *C. krusei* (Trofa et al., 2008; Van Asbeck et al., 2009; Tóth et al., 2019).

Complexo *Candida parapsilosis* é a segunda espécie de *Candida* mais isolada na Ásia, Sul da Europa e América Latina chegando inclusive a superar a frequência de *Candida albicans* em alguns hospitais pediátricos. Dependendo da localização geográfica, este patógeno pode ser o principal agente a causar infecções sanguíneas invasivas (candidemias) (Cattana et al., 2017; Branco et al., 2023).

O crescente aumento do envolvimento de espécies de CNA em candidíases humanas poderia ser relacionado, pelo menos em parte, ao melhoramento dos métodos diagnósticos, tais como o uso de ágar para isolamento primário com a habilidade de diferenciar as espécies de *Candida* e a introdução de técnicas de diagnóstico molecular na rotina (Liguori et al., 2009; Friedman et al., 2019). Outros fatores poderiam ser implicados no aumento da prevalência de espécies de *Candida*, incluindo a introdução e difusão de certas práticas médicas, tais como terapia imunossupressora, uso de antibióticos de amplo espectro e aumento no número de transplantes de órgãos e de células tronco hematopoiéticas (Kojic and Darouichee, 2004). Além disso, o aumento do número de espécies de *Candida* em candidíases poderia ser um reflexo da seleção de espécies na presença de certos antifúngicos, visto o maior nível de resistência

demonstrada para várias espécies de CNA (Gravina et al., 2007; Whaley et al. 2016; Xiao 2018).

1.2 COMPLEXO *CANDIDA PARAPSILOSIS*

C. parapsilosis foi isolada em 1928, em um paciente em Porto Rico com diarreia. Naquela época foi classificada como *Monilia parapsilosis*, sendo assim considerada do gênero *Monilia*, devido a sua incapacidade de fermentar maltose (Ashford, 1928). A espécie foi nomeada *Monilia parapsilosis* para distinguir do isolado mais comum, *Monilia psilosis*, conhecida hoje como *C. albicans* (Trofa et al.,2008). Desde então, essa espécie vem sofrendo várias alterações na sua taxonomia.

Anos após seu isolamento, *C. parapsilosis* foi separada em três grupos, I, II e III, de acordo com dados fenotípicos e moleculares (Lott et al.,1993), até que em 2005 Tavanti e colaboradores, com base em estudos genéticos moleculares [sequenciamento em multilocus (MLST) – (11 genes: ACPL, ACPR, COX3, GAL1, L1A1, LIP2, SADH, SAPP3, SYA1, TOP2 e URA3)], demonstraram a existência de diferenças significativas entre os grupos. Diante disso, sugeriram que os grupos II e III de *C. parapsilosis* constituíssem novas espécies, sendo nomeadas *Candida orthopsilosis* e *Candida metapsilosis*, respectivamente, mantendo o grupo I como *C. parapsilosis stricto sensu*. O grupo dessas espécies também é frequentemente referido como *Candida parapsilosis sensu lato*, sendo que a distinção das espécies só pode ser realizada por meio de técnicas moleculares.

As espécies do complexo *C. parapsilois* são responsáveis por uma ampla variedade de manifestações clínicas, sendo consideradas agentes patogênicos oportunistas. As infecções causadas por estas espécies vão desde doenças invasivas, como candidemia, endocardite e peritonite, que geralmente ocorrem em associação com procedimentos invasivos ou dispositivos médicos, até infecções superficiais das mucosas, pele e unhas (Cantón et al., 2011; Garcia-Effron et al., 2012; Levy et al., 1998; Miranda et al., 2012; Ruiz et al., 2013; van Asbeck et al., 2007).

Na década de 1970, *C. parapsilosis* ganhou importância devido ao aumento no número de casos de candidíases causadas por essa espécie. Um exemplo, é a candidíase em neonatos prematuros, uma vez que esses possuem integridade da pele comprometida e são mais suscetíveis por utilizarem cateteres por longos períodos (Benjamin et al., 2004; Bonassoli et al.,2005; Nemeth et al., 2018).

As espécies do Complexo *C. parapsilosis* tem atraído atenção como agente de infecções fúngicas invasivas, principalmente entre neonatos com baixo peso ao nascer, indivíduos imunocomprometidos e pacientes que necessitam de uso prolongado de cateteres venosos centrais (Branco et al., 2023).

De acordo com vários estudos, dentre as espécies do complexo *C. parapsilosis*, *C. parapsilosis* sensu stricto é a mais prevalente em infecções humanas. Borghi et al. (2011) demostraram taxas de prevalência de 95%, 3,6% e 1,4% para *C. parapsilosis sensu stricto*, *C. orthopsilosis* e *C. metapsilosis*, respectivamente, em infecções fúngicas invasivas. Feng et al. (2012) descreveram resultados semelhantes, porém para candidíase superficial, onde a *C. parapsilosis sensu stricto* representou 96,5% dos isolados, seguida pela *C. metapsilosis* (2.5%) e *C. orthopsilosis* (1.0%). No ano seguinte, Tosun et al. (2013) avaliaram a distribuição das espécies do complexo em amostras clínicas oriundas tanto de infecções superficiais quanto de infecções profundas, e demostraram que *C. orthopsilosis* e *C. metapsilosis* juntas representavam menos de 10% dos agentes etiológicos destas infecções. Um estudo publicado pelo nosso grupo mostrou resultados semelhantes, ou seja, uma alta prevalência de *C. parapsilosis* sensu stricto (95%) entre as espécies do complexo em pacientes de Uberaba, MG (Silva et al., 2015).

Um estudo publicado por pesquisadores da Índia, mostraram que dentre as espécies do complexo, *C. parapsilosis* sensu stricto era a espécie mais comum (51,1%), porém foi identificada uma alta prevalência de *C. orthopsilosis* (40,2%) em amostras clínicas (Maria et al., 2018).

Apesar desses dados disponíveis, a caracterização das espécies do complexo *C. parapsilosis* ainda não está concluída, ressaltando a importância de mais estudos para compreender a distribuição de cada membro do complexo, especialmente *C. orthopsilosis* e *C. metapsilosis* (Silva et al., 2015).

A nutrição parenteral, emulsão lipídica intravenosa e intubação são os principais fatores de risco para a doença neonatal por *C. parapsilosis* (Saiman et al., 2000). Nesses pacientes, a infecção pode afetar quase todos os locais do corpo, incluindo pulmões, urina, retina e sistema nervoso central (Carter et al., 2008).

Em um estudo publicado em 2013, focando especificamente a candidemia neonatal, os autores demostraram que *C. parapsilosis* correspondia a 33,47% de todas as infecções neonatais por *Candida*. A infecção não apresentou distribuição geográfica homogênea, variando entre 19,1 e 35,8% entre os continentes (Europa 19,1%, Ásia 24,7%, América do Sul 29,1%, América do Norte 33,8% e Austrália 35,8%) (Pammi et al., 2013).

Dentre as espécies de *Candida*, *C. albicans* é a mais prevalente nas micoses superficiais. Porém, estudos mostram que *C. parapsilosis* vem desempenhando um papel importante como agente etiológico dessas micoses, em particular das onicomicoses, sendo, inclusive, algumas vezes implicada como a espécie de *Candida* mais comumente associada às onicomicoses em algumas regiões (Mugge et al., 2006; Fich et al., 2014).

Segundo Ataides et al. (2012), em um estudo realizado em Goiânia, GO, a espécie mais frequente nas onicomicoses foi C. *parapsilosis*, representando 53,4% das leveduras isoladas dessas lesões. Resultados semelhantes, foram verificados pelo nosso grupo, ou seja, uma alta prevalência de *C. parapsilosis* entre os agentes etiológicos de dermatomicoses, particularmente onicomicoses, em pacientes de Uberaba, MG (Silva et al., 2014).

A candidíase vulvovaginal (CVV) é uma infecção comum, afetando 50% das mulheres em idade fértil, podendo ser recorrente (Trofa et al., 2008). É causada principalmente por *C. albicans*, porém é cada vez mais comum espécies de *Candida* não *albicans*, como C. *parapsilosis* causar CVV, devido ao uso excessivo ou até mesmo indevido de antifúngicos (Zhang et al.,2014).

As espécies de *Candida* são os agentes mais comuns envolvidos na endocardite fúngica, seguidas pelas espécies de *Aspergillus* (Abgueguen et al., 2002) e a *C. parapsilosis* é identificada em 17% dos casos, com uma taxa de mortalidade de aproximadamente 50%, tornando-se a segunda espécie mais comum de *Candida*, após *C. albicans* (Garzoni et al., 2007).

Na Europa, *C. parapsilosis* já é responsável por 20% a 25% das infecções invasivas por *Candida* spp. (Vogiatzi et al., 2013; Tedeschi et al., 2016). Estudos mostram que na Sérvia houve um aumento na prevalência de infecções por *C. parapsilosis*, sendo esta espécie responsável por 46% dos casos de candidíases em adultos, o que é semelhante à frequência de *C. albicans* (Arsenijevic´et al., 2018).

Na Ásia e na América do Norte, a prevalência de *C. parapsilosis* depende da região. *C. parapsilosis* é a segunda espécie mais frequentemente identificada em ambiente hospitalar no Japão e na China, sendo responsável por 20 a 23% de todas as infecções da corrente sanguínea por *Candida*; enquanto é a terceira espécie do gênero *Candida* mais comumente identificada na Índia e outras regiões tropicais da Ásia (Tan et al., 2015; Xiao et al., 2018).

Dentre as espécies CNA, a *C. parapsilosis* é frequentemente isolada na América do Sul, se destacando em algumas regiões como Colômbia (36,7% a 38,5%) e Venezuela (26,8% a 39%), superando até mesmo as infecções por *C. albicans* (Bustamante et al., 2014; Nucci et al., 2018).

Um estudo publicado em 2016 de Govender e colaboradores, relata o aumento da incidência de candidemias causadas por *C. parapsilosis* na África do Sul, sendo esta espécie responsável por 35% dos casos de candidemias em hospitais públicos, estabelecendo-se como a segunda espécie mais comum. No setor privado, por sua vez, esse número chega a 50%, superando assim as infecções causadas por *C. albicans* (Govender et al., 2016). Além disso, está espécie é responsável por 15% dos casos de candidemias nos Estados Unidos (Lamoth et al., 2018) e 20% na Rússia (Vasilyeva et al., 2018).

1.3 FATORES DE VIRULÊNCIA

As infecções por espécies do complexo *C. parapsilosis* são facilitadas por um conjunto de fatores de virulência, que vão contribuir na colonização, invasão e patogênese do fungo (Naglik et al., 2003; Tamura et al., 2007). A despeito de importantes pesquisas objetivando identificar esses fatores de virulência em espécies de *Candida*, relativamente pouco é conhecido sobre os determinantes de virulência nas espécies do complexo *C. parapsilosis.* Além disso, os poucos dados existentes sugerem que as três espécies podem apresentar diferentes potenciais de virulência. Por exemplo, Orsi et al. (2010) compararam o potencial patogênico entre as espécies do complexo *C. parapsilosis* utilizando um modelo de infecção *in vitro* em micróglia.

Esses autores demostraram que a *C. metapsilosis* é menos virulenta, quando comparada com as demais espécies do complexo. Anos mais tarde, outro estudo realizado por Németh et al. (2013) e utilizando um modelo *in vivo* com larvas de *Galleria mellonella* confirmaram a menor virulência da *C. metapsilosis* em relação às outras espécies do complexo. Gago e colaboradores, tiveram resultados semelhantes, e demostraram que a *Candida metapsilosis* promovia uma taxa de mortalidade larval bem menor do que aquela observada para as demais espécies do complexo (Gago et al.,2014).

Dentre os fatores reconhecidos como determinantes para a virulência das espécies do complexo *Candida parapsilosis*, pode-se destacar a habilidade de formação de biofilme e a produção de exoenzimas (esterase, fosfoçipase, hemolisina e proteinases) (Gácser et al., 2007).

1.3.1 Formação de biofilme

Biofilmes são agregados de microrganismos associados a uma superfície e incrustados em uma matriz extracelular (Trofa et al., 2008). Eles são considerados a forma mais prevalente de crescimento microbiano na natureza e frequentemente estão associados com infecções clínicas. É um importante fator de virulência para várias espécies de *Candida* (Tumbarello et al., 2007; Trofa et al., 2008; Nobile et al., 2015).

A formação do biofilme inicia-se com a aderência dos microrganismos em tecidos (superfície biótica) ou dispositivos médicos (superfície abiótica), sendo o mais comum o cateter venoso central. Após a fixação, as células fúngicas proliferam rapidamente, formando assim uma monocamada. Depois da aderência, as células de leveduras passam por uma transição morfológica, formando pseudo-hifas e hifas (em *C. albicans*), seguindo para o desenvolvimento do biofilme, caracterizado pela produção de uma matriz extracelular composta por substâncias inorgânicas e orgânicas, na qual são encontradas todas as quatro principais classes de macromoléculas (polissacarídeos, proteínas, lipídios e ácidos nucléicos). Os elementos da matriz fornecem estabilidade estrutural e funcional ao biofilme. Uma vez que o estágio de biofilme atinge uma estrutura complexa e estável, as células fúngicas são protegidas e aguardam condições ambientais benéficas para iniciar a dispersão (Seneviratne et al., 2008; Trofa et al., 2008).

O biofilme proporciona muitos benefícios aos microrganismos que os constitui, tais como: aquisição de nutrientes, os quais estão presentes na matriz polimérica; proteção contra desidratação e outros fatores ambientais adversos (Ramage et al., 2001; Trofa et al., 2008); possibilita a troca de material genético e a capacidade de colonizar diferentes nichos ecológicos (Flemming,1993; Mittelman, 1998). Além disso, fornece proteção contra antifúngicos, bem como proteção contra as células e mediadores solúveis do sistema imune do hospedeiro (Nett, 2016).

Os biofilmes de *C. parapsilosis* são menos complexos e mais finos do que aqueles de *C. albicans*. Em termos estruturais, os biofilmes de *C. parapsilosis* consistem principalmente de blastoconídeos agregados e pseudo-hifas, sendo que os blastoconídeos se apresentam em um agrupamento irregular numa camada basal (Ferreira et al., 2019). Além disso, o biofilme possui uma grande quantidade de carboidratos e uma pequena quantidade de proteínas, sendo que a formação do biofilme por esta espécie é totalmente dependente da estirpe (Kuhn et al., 2002; Silva et al., 2009).

Estudos demostram que há diferenças entre as espécies do complexo *C. parapsilosis* quanto à capacidade de formação de biofilme. Tavanti et al. (2010) relataram que 64.5% das amostras de *C. parapsilosis sensu stricto* foram capazes de formar biofilme. Toro et al. (2011) mostraram que 58.5% dos isolados de *C. parapsilosis* sensu stricto foram capazes de formar biofilme, enquanto nenhum dos isolados clínicos de *C. orthopsilosis* e *C. metapsilosis* produziu biofilme *in vitro*. Dados semelhantes foram encontrados por Tosun et al. (2013). Por outro lado, existem dados que demonstram que as três espécies do complexo são capazes de produzir biofilmes (Melo et al., 2011; Abi-chacra et al., 2013). Isso também foi verificado em um estudo recentemente publicado, onde os autores descrevem que as três espécies são produtoras de biofilme. Ao mesmo tempo, os autores verificaram que *C. metapsilosis* produz biofilme mais rápido do que as demais espécies, em média após 48h (Modiri et al., 2019).

No entanto, vale ressaltar que vários desses estudos mencionados não são diretamente comparáveis porque eles diferem em aspectos importantes, tais como o processo utilizado para analisar a capacidade de formação do biofilme, os métodos para detectar a produção do biofilme (ou seja, coloração com cristal violeta, ensaios de redução de XTT e medida da transmitância ou absorbância sem coloração) e os critérios para considerar um isolado como produtor ou não produtor de biofilme.

1.3.2 Esterase

As lipases extracelulares são importantes fatores de virulência das espécies do gênero *Candida* (Silva et al., 2012). Estas enzimas lipolíticas têm a função de catalisar a hidrólise e a síntese de triacilgliceróis resultando na liberação de ácidos graxos (Trofa; Gacser; Nosanchuk, 2008; Silva et al., 2012, Toth et al., 2017). Desse modo, elas desempenham papel fundamental na digestão de lipídeos, o que resulta na aquisição de nutrientes, interação sinérgica com outras enzimas, adesão às células e tecidos do hospedeiro, ativação inespecífica de processos inflamatórios e lise da microbiota competidora (Trofa; Gacser; Nosanchuk, 2008; Sardi et al., 2013).

Dentre as lipases extracelulares, destacam-se as esterases, que atuam sobre compostos mais solúveis em água e de cadeia molecular curta (Schaller et al., 2005). Em *C. parapsilosis* as lipases extracelulares parecem ser responsáveis pela destruição tecidual. Gácser e colaboradores demostraram que o uso de inibidores de lipases durante a infecção por esta espécie levou à redução da lesão tecidual (Gácser et al., 2007). Treviño-Rangel e colaboradores (2013) mostraram que 67% dos isolados de *C. orthopsilosis* e 13% de *C. parapsilosis* sensu

stricto apresentaram alta atividade lipolítica e *C. metapsilosis* não apresentou secreção extracelular de lipases, evidenciando ser a espécie menos virulenta do complexo (Orsi et al., 2010; Németh et al., 2013). Entretanto, alguns estudos relataram que todas as espécies do complexo *C. parapsilosis* são capazes de produzir esterases (Ge et al., 2011; Ziccardi et al., 2015).

1.3.3 Fosfolipase

As fosfolipases são enzimas que frequentemente estão associados com a patogenicidade de espécies de *Candida* (Price et al.,1982). Sua função durante a infecção é hidrolisar constituintes lipídicos da membrana celular do hospedeiro, o que acarreta danos celulares e/ou exposição de receptores que facilitariam a aderência e a invasão (Ghannoum, 2000; Mohan das; Ballal, 2008; Silva et al., 2012).

As espécies do complexo *C. parapsilosis* secretam fosfolipases, embora com ampla variação entre as espécies, com estudos relatando que 9 a 90% dos isolados clínicos produzem essas enzimas (D'Eça Junior et al., 2011; Silva et al., 2015). Dentre esses estudos, podemos destacar Abi-chacra e colaboradores (2013), que encontraram atividade baixa ou indetectável de fosfolipases nas espécies do complexo *C. parapsilosis.* Por outro lado, Trevino-Rangel e colaboradores, no mesmo ano, relataram a atividade dessas enzimas em 97% (29/30) dos isolados de *C. orthopsilosis*, 80% (4/5) dos *C. metapsilosis* e 63% (19/30) dos *C. parapsilosis* sensu stricto (Trevino-Rangel et al., 2013).

Pharkjaksu e colaboradores relataram atividade para fosfolipases em 90,91% (60/66) dos isolados de *C. parapsilosis* sensu stricto, 42,86% (12/28) de *C. orthopsilosis* e 100% (2/2) de *C. metapsilosis* (Pharkjaksu et al., 2018). No entanto, sua contribuição para a patogenicidade ainda é motivo de debate, pois não há uma relação direta entre sua expressão e o grau de virulência. Contudo, é geralmente aceita a ideia de que a elevada produção dessas enzimas pode estar relacionada a um aumento dos níveis de patogenicidade das leveduras, já que amostras que expressam altas quantidades de fosfolipases apresentam uma maior capacidade de aderência e invasão nas células do hospedeiro (Ibrahim et al., 1995; Ghannoum, 2000; Kantarcioglu et al., 2002).

A variação na expressão de fosfolipases nas espécies do complexo *C. parapsilosis* pode estar relacionada ao uso de diferentes testes enzimáticos, ao tamanho da amostra e/ou variação biológica entre os isolados (Ge et al., 2012).

1.3.4 Hemolisina

As hemolisinas são uma classe de proteínas que tem a habilidade de lizar as células vermelhas do sangue, consequentemente ocorre a degradação de hemoglobina e/ou do grupo heme, resultando na liberação de ferro (Giolo; Svidzinski, 2010; Nayak; Green; Beezhold, 2013). O ferro é um elemento essencial para o desenvolvimento das leveduras, pois contribui para o crescimento fúngico, atua nos processos metabólicos e como catalisador de vários processos bioquímicos (Nayak; Green; Beezhold, 2013; Rossoni et al., 2013) sendo, portanto, fundamental para o estabelecimento da infecção, uma vez que contribui para sobrevivência das leveduras e para invasão de hifas durante o processo infeccioso (Favero et al., 2011; Silva et al., 2012; Nayak; Green; Beezhold, 2013; Rossoni et al., 2013; Sardi et al., 2013).

Um estudo realizado por Favero et al., (2013) demonstrou que as amostras de *C. parapsilosis* não tinham nenhuma atividade para essa enzima, porém, um ano após esse estudo, Ziccardi e colaboradores descreveram que as espécies do complexo *C. parapsilosis* apresentaram atividade hemolítica. No referido estudo foram avaliadas 53 amostras, sendo 43 identificadas como *C. parapsilosis* sensu stricto e 10 como *C. orthopsilosis*, dentre as quais 13 e 4 apresentaram atividade hemolítica, respectivamente. (Ziccardi et al., 2015).

Mais recentemente, Ferreira et al., (2019) avaliaram a atividade hemolítica de amostras do complexo *C. parapsilosis* e observou-se que as três espécies do complexo foram capazes de produzir atividade para essa enzima.

1.3.5 Proteinase

As aspartil proteases estão presentes em vários microrganismos e desempenham um papel crucial na patogênese e na aquisição de nutrição. As proteinases (Saps) são reconhecidas como importantes determinantes de virulência para *Candida*, especialmente *C. albicans* (KogaIto et al., 2006; Ruchel et al., 1982). *Candida parapsilosis* possui três genes codificadores da proteína SAPP (SAPP1, SAPP2, SAPP3) que podem contribuir para a virulência (Merkerová et al., 2006; Singh et al., 2019).

Essas proteinases facilitam a colonização e invasão dos tecidos do hospedeiro por meio do rompimento das membranas mucosas do hospedeiro e pela degradação de importantes proteínas de defesa imunológica (Pichova et al., 2001).

Um estudo realizado por Tosun et al. (2013) demostrou que 34,2% (29/77) dos isolados de *C. parapsilosis* sensu stricto expressaram proteinases. Além disso, nenhum dos seus isolados de

C. metapsilosis ou *C. orthopsilosis* apresentaram atividade para essa exoenzima. No entanto, Trevino-Rangel et al. (2013) relataram que 17% (5/30), 7% (2/30) e 60% (3/5) dos isolados de *C. parapsilosis* sensu stricto, *C. orthopsilosis* e *C. metapsilosis*, respectivamente, produziram proteinases.

Outros estudos mostram uma alta prevalência da expressão de proteinases em isolados de *C. parapsilosis* sensu stricto (Abi-chacra et al., 2013; Brilhante et al., 2018). De modo similar, existem relatos que encontraram também elevada porcentagem de expressão dessas enzimas entre isolados de *C. metapsilosis* e *C. orthopsilosis* (Ge et al., 2011; Németh et al., 2013;).

De acordo com Ge et al. (2011), as razões para essa variabilidade entre os estudos são desconhecidas, mas pode ser devido aos meios utilizados para os testes enzimáticos, pois a sensibilidade dos meios utilizados nos ensaios varia; outra razão pode estar relacionada com as diferentes temperaturas de incubação utilizadas nos ensaios.

1.4 TERAPIA ANTIFÚNGICA E MECANISMO DE AÇÃO

Apesar do desenvolvimento das pesquisas em relação à terapia antifúngica, poucas são as classes de antimicóticos disponíveis para o tratamento de infecções fúngicas. Na realidade, as células fúngicas, quando comparadas com as células humanas, têm uma grande similaridade bioquímica e fisiológica, que dificulta a obtenção de fármacos com toxicidade seletiva para os fungos.

Atualmente, na prática clínica para o tratamento de infeções fúngicas sistémicas, incluindo infeções por espécies do complexo *C. parapsilosis*, os antifúngicos podem ser divididos em quatro classes principais: os azóis, as alilaminas, os polienos e as equinocandinas (Pippi et al., 2015).

Os azóis (ex. fluconazol, itraconazol e voriconazol), atuam inibindo a síntese de ergosterol na membrana plasmática fúngica, por meio da inibição da enzima esterol 14desmetilase. Nessa classe, o fluconazol (FLC) se destaca, por ser o antifúngico mais prescrito atualmente. É utilizado como profilaxia em várias situações, especialmente para prevenir infecções fúngicas em pacientes imunocomprometidas (pacientes com doenças malignas, recém nascidos prematuros e pacientes em unidades de terapia intensiva), ainda pode ser utilizado como tratamento de infecções por espécies de *Candida* (Pfaller et al., 2010; Santos et al., 2018).

Os polienos (ex. anfotericina B) agem na membrana celular fúngica, ligando-se ao ergosterol e alterando a integridade da membrana plasmática. Por possuir ação fungicida e é considerada um medicamento com ação de amplo espectro contra fungos dimórficos, filamentosos e leveduras, a anfotericina B é utilizada para a maioria das infecções sistêmicas, entre elas, candidíase invasiva, aspergilose e criptococose (Odds et al., 2003). A anfotericina B (AMB), apesar de eficaz, apresenta alguns desafios na terapia convencional, incluindo sua baixa solubilidade em água e alta toxicidade. Uma alternativa para substituir a AMB poderia ser o FLC (Ostrosky-Zeichner et al., 2003; Kuhn et al., 2004).

Quanto às equinocandinas [caspofungina (CAS), micafungina e anidulafungina] a mais nova classe de agentes antifúngicos, têm como alvo a parede celular de fungos pela inibição da enzima β-1,3-D-glucana sintase, que catalisa a síntese de glucanas que a compõem, conduzindo à instabilidade osmótica e lise celular. São consideradas uma ótima escolha para tratamento de infecções sistêmicas por espécies do complexo *C. parapsilosis* (Odds et al., 2003).

Embora infecções por *C. parapsilosis* geralmente resultem em taxas de morbidade e mortalidade mais baixas do que infecções causadas por *C. albicans*, vários isolados clínicos do complexo *C. parapsilosis* foram relatados como menos sensíveis às equinocandinas e, em algumas regiões, também foi observada resistência ao tratamento com azóis, o que complica a escolha da terapia com antimicóticos (Tóth et al., 2019; Lass-Flörl et al., 2024).

1.5 RESISTÊNCIA AOS ANTIFÚNGICOS

O desenvolvimento de resistência aos agentes antifúngicos é considerado um importante causa de falha do tratamento de infecções fúngicas. Se pudermos escolher de forma inteligente um antifúngico de acordo com o agente etiológico, a eficácia da terapia contra as micoses poderia ser melhorada (Gupta & Kohli, 2003). Para isso, é de fundamental importância conhecer o perfil de sensibilidade dos fungos aos agentes antifúngicos. Nesse contexto, sugere-se que a menor sensibilidade das leveduras do complexo *C. parapsilosis* aos azóis e equinocandinas pode tornar-se um motivo de preocupação clínica (Cantón et al., 2011; Moudgal et al., 2005; Sarvikivi et al., 2005).

O uso profilático e empírico dos agentes antifúngicos das diferentes classes, associado ao aumento de procedimentos médicos invasivos e da população de pacientes imunossuprimidos, podem estar relacionados ao desenvolvimento de resistência aos antifúngicos, estando os microrganismos muitas vezes expostos a concentrações subinibitórias de antifúngicos (Presterl et al., 2007; Tobudic et al., 2012; White et al., 2015).

Além disso, o uso indiscriminado de antifúngicos favorece o surgimento de cepas resistentes aos medicamentos entre populações anteriormente suscetíveis. Vale ressalta que, a exposição prolongada a antifúngicos, pode desenvolver isolados de espécies de *Candida* resistentes, evidenciando que são microrganismos altamente adaptáveis (Brilhante et al., 2019; Fekete-Forgács et al., 2000; Martínez et al., 2022; Paul et al., 2020; Pinto e Silva et al., 2009; Rocha et al., 2016).

A aquisição de resistência antifúngica possibilita a neutralização dos efeitos fungicidas ou fungistáticos das diferentes classes de antifúngicos e isso pode ser alcançado por meio de três mecanismos principais: redução do acúmulo do fármaco no interior da célula fúngica; diminuição da afinidade do antifúngico pelo seu alvo; modificações do metabolismo para contrabalançar o efeito do fármaco (Vandeputte et al., 2012). Dentre as classes dos antifúngicos, os azóis são os mais utilizados na clínica e, consequentemente, são os mais investigados (Chau et al.,2005; Jiang et al., 2013, Neji et al., 2017).

O surgimento de isolados de *C. parapsilosis* resistentes ou com a sensibilidade diminuída ao (FLC) pode estar relacionado ao uso excessivo desse antifúngico para profilaxia e tratamento de infecções fúngicas além disso, a baixa adesão dos profissionais de saúde aos protocolos de antissepsia das mãos pode contribuir para disseminação das cepas resistentes aos antifúngicos (Almirante et al., 2006; Govender et al., 2016). Outro fator importante, é em relação ao aumento da capacidade de amostras resistentes à FLC e CAS formarem biofilme (Lass-Flörl et al., 2024).

Um estudo em 2011 relatou sobre a frequência de resistência antifúngica entre pacientes com infecção na corrente sanguínea, internados em Unidade de Terapia Intensiva (UTI) e fora da UTI por espécies de *Candida*. Dentre as espécies de *Candida* foram identificadas 118 amostras de *C. parapsilosis* em pacientes da UTI, sendo 6,8% resistentes a fluconazol e das 184 amostras identificadas como *C. parapsilosis* de pacientes não internados em UTI, obtiveram 4,3% amostras resistente a fluconazol (Pfaller et al., 2011). Govender e colaboradores 2016, demostraram que apenas 37% de um total de 531 amostras de *C. parapsilosis* eram sensíveis a fluconazol e voriconazol. Estudos mostram que a resistência aos azóis é mais comum que a não sensibilidade às equinocandinas. Em 2018 foi publicado um trabalho, em que os autores avaliaram 44 amostras de *C. parapsilosis* sensu stricto, sendo 13,63% resistentes ao fluconazol e 4,54% com sensibilidade dose-dependente (SDD). Além disso, o estudo demostrou oito amostras com sensibilidade dose-dependente para o voriconazol, 8 (18,18%), de um total de 44 amostras (Maria et al., 2018).

Dados semelhante foram encontrados por Meletiadis et al. (2017), que avaliaram 122 amostras de *C. parapsilosis* sensu stricto, sendo 9.8% resistentes ao fluconazol e 5.8% resistentes ao voriconazol. Em relação às equinocandinas, apenas três amostras apresentaram sensibilidade intermediária para anidulafungina e uma amostra com sensibilidade intermediária para micafungina (Meletiadis et al., 2017). Desses trabalhos, nenhum deles demostrou resistência em isolados de *C. metapsilosis* e *C. orthopsilosis*, ao contrário dos resultados de pesquisas anteriores (Chen et al.,2010; Feng et al., 2012; Tosun et al., 2013).

1.6 *GALLERIA MELLONELLA*

Como discutido anteriormente, as espécies do complexo *C. parapsilosis* são agentes etiológicos importantes de infecções superficiais e sistêmicas. Com o aumento dessas infecções fúngicas e também o aumento do isolamento de espécies resistentes oriundas de amostras clínicas, há necessidade de esclarecer se a aquisição de resistência pode afetar as características de virulência dessas espécies e, consequentemente, suas relações com o hospedeiro. Nos últimos anos, tem-se buscado modelos experimentais para testes com patógenos humanos, eticamente mais aceitáveis que os modelos em mamíferos. Os modelos murinos são o padrão ouro para estudos de virulência. No entanto, modelos experimentais em hospedeiros invertebrados, como a *Galleria mellonella*, tem se tornado uma alternativa atraente, pois esses modelos são simples e de baixo custo (Chamilos et al., 2007; Borghi et al., 2014). Várias pesquisas têm mostrado a utilidade de *G. mellonella* como modelo para estudar diversos aspectos da patogenicidade dos fungos (Brennan et al., 2002; Fuchs et al., 2010; Junqueira et al., 2012; Németh et al., 2013; Perini et al., 2019).

As larvas do inseto *G. mellonella* oferecem vantagens importantes em relação a modelos mamíferos, como: possibilidade de manutenção larval em uma ampla faixa de temperatura (2537°C), característica relevante para estudar as propriedades das espécies fúngicas, incluindo *Candida* spp., quando crescidas em diferentes temperaturas; utilização de instalações simples para sua manutenção; fácil manejo e inoculação de microrganismos; a ausência de requerimento de equipamentos especiais para avaliação da mortalidade (Fuchs et al., 2010; Gago et al., 2014). Além disso, as larvas de *G. mellonella* apresentam um sistema imunológico que possui semelhança ao sistema imune inato dos mamíferos. Os hemócitos, células de defesa da larva contra bactérias e fungos, atuam como fagócitos, semelhantes aos neutrófilos nos seres humanos (Gago et al., 2014).

Assim, as larvas *G. mellonella* podem ser utilizadas como um modelo experimental de infecção para entender a aquisição de resistência a determinados antifúngicos. Avaliando como a resistência pode afetar características de virulência das espécies do complexo *C. parapsilosis*, de maneira fácil e confiável.

1.7 CONSIDERAÇÕES FINAIS

As infecções humanas causadas por espécies do complexo *C. parapsilosis* resistentes a antifúngicos vêm se tornando mais frequentes, representando um grande risco à saúde pública. Em parte, podemos atribuir o aumento da resistência ao uso indiscriminado dos antimicóticos, que contribui para um processo de seleção natural, resultando na ocorrência de cepas mais resistentes. Ao mesmo tempo, há um aumento no número de pacientes imunossuprimidos e de procedimentos médicos invasivos, predispondo, consequentemente, um maior número de indivíduos a desenvolver infecções oportunistas por espécies do complexo *C. parapsilosis*.

Diante disso, ressaltamos a necessidade de pesquisas que busquem compreender as consequências do desenvolvimento de resistência a antifúngicos específicos nas características de virulência dessas espécies, bem como o potencial de resistência cruzada a diferentes classes de antifúngicos. Em suma, esses foram os objetivos principais da pesquisa desenvolvida durante o trabalho de doutorado apresentado no artigo a seguir, onde fornecemos uma compreensão sobre as respostas adaptativas de espécies do complexo *C. parapsilosis* à pressão antifúngica, destacando a intrincada interação entre mecanismos de resistência e potencial patogênico.

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2 ARTIGO SUBMETIDO

In Vitro Induction of Resistance to Fluconazole and Caspofungin in *Candida parapsilosis* Complex Species: Implications for Virulence and Cross-Resistance to Other Antifungals.

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Abstract

This study investigates the effects of progressive exposure to fluconazole (FLC) and caspofungin (CSF) on resistance development and virulence traits in the *Candida parapsilosis* complex. Seventeen isolates (6 *C. parapsilosis* sensu stricto, 6 *C. orthopsilosis*, and 5 *C. metapsilosis*) were exposed to increasing concentrations of FLC and CSF, and minimum inhibitory concentrations (MICs) were determined pre- and post-exposure. Significant increases in MICs for FLC and CSF were observed. Notably, induced resistance to one antifungal agent influenced susceptibility to other antifungals, with an inverse relationship between the MICs of azoles (FLC, itraconazole, and voriconazole) and echinocandins (CSF, anidulafungin, and micafungin). Additionally, there was a significant increase in amphotericin B MICs in samples with induced resistance to both FLC and CSF. Resistance phenotypes remained largely stable after subculturing in antifungal-free media. Antifungal exposure enhanced biofilm formation but did not significantly affect the secretion of key hydrolytic enzymes (esterase, phospholipase, protease, and haemolysin). *In vivo* virulence remained unchanged post-resistance induction, as assessed using the *Galleria mellonella* model. Our findings highlight the adaptability of *C. parapsilosis* complex species to antifungal pressure, underscoring the need for continuous surveillance and the development of novel therapeutic strategies to manage infections caused by these pathogens. The increase in biofilm formation following antifungal exposure presents additional challenges for infection management.

Keywords: *Candida parapsilosis* complex; Antifungal resistance; Fluconazole; Caspofungin; Biofilm formation; Virulence traits.

Highlights

- Fluconazole and caspofungin resistance were experimentally induced in *C. parapsilosis* complex isolates.
- Cross-resistance to azoles and echinocandins was observed in isolates post-resistance induction. An inverse relationship between azole and echinocandin MICs was observed post-resistance induction.
- Resistance acquisition did not significantly affect the secretion of key hydrolytic enzymes.
- Resistance acquisition correlated with increased biofilm formation.

Introduction

The genus *Candida* frequently causes human diseases, ranging from superficial to lifethreatening invasive infections. Within this genus, *Candida albicans*, which is usually susceptible to antifungals, has historically been the most prevalent species recovered from clinical samples. However, with the increasing use of antifungal agents and the development of new diagnostic techniques, a shift in the epidemiology of *Candida* infections is occurring. This shift has led to a rise in infections caused by non-*albicans Candida* species, which have less predictable antifungal susceptibility (Friedman et al., 2019).

Among non-*albicans Candida* species, the *Candida parapsilosis* complex, comprising *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*, has garnered attention as an agent of invasive fungal infections, particularly prevalent among neonates with low birth weights, immunocompromised individuals, and patients requiring prolonged use of central venous catheters (Branco et al., 2023). Additionally, this complex is an important etiological agent of superficial mycoses, such as onychomycoses (da Silva et al., 2015; Silva et al., 2014. Among these three sibling species, *C. parapsilosis* sensu stricto is the most prevalent infectious agent (Lass-Flörl et al., 2024).

Candida parapsilosis is the second most common *Candida* species isolated in Asia, Southern Europe, and Latin America (Branco et al., 2023). Depending on the geographical location, this pathogen can be the primary agent for non-*C. albicans* invasive blood infections (candidemia). Due to its clinical significance, *C. parapsilosis* is included among the fungi in the first Fungal Priority Pathogens List (FPPL) released by the World Health Organization (WHO), ranking in the high priority group (WHO, 2022).

Compared to other *Candida* species, the *C. parapsilosis* complex exhibits intrinsically high minimal inhibitory concentrations (MICs) for echinocandins (comprising caspofungin (CSF), micafungin (MCF), and anidulafungin (ANF)), one of the three classes of systemic antifungal medicines available for treating *C. parapsilosis*-related infections, along with azoles and polyenes. Furthermore, *C. parapsilosis* infections have become particularly concerning in the past few years due to a significant rise in resistance to fluconazole (FLC), one of the most common azoles used for systemic *Candida* infections, with resistance increasing more markedly and acutely in *C. parapsilosis* than in any other species (Lass-Flörl et al., 2024).

Despite being an increasing global public health concern, the biology of *C. parapsilosis* complex species has not been as extensively explored as that of *Candida albicans* (Branco et al., 2023), particularly for *C. orthopsilosis* and *C. metapsilosis* (Brilhante et al., 2018). The propensity of these complex species to develop resistance to commonly used antifungal agents reveals their adaptability to various antifungal pressures and underscores the need for further studies. These studies should aim to understand the consequences of resistance development to specific antifungals on virulence characteristics, as well as the potential for cross-resistance to other antifungals in these species.

Our study aimed to investigate the impact of progressive exposure to FLC and CSF on the antifungal susceptibility profiles of *C. parapsilosis* complex isolates. We focused on changes in MIC values, the potential for cross-resistance, and the stability of resistance phenotypes after subculturing in drug-free media. Additionally, we assessed the effects of induced resistance on key virulence factors, including hydrolytic enzyme production and biofilm formation, as well as *in vivo* virulence using the *Galleria mellonella* model. Our findings provide insights into the adaptive responses of *C. parapsilosis* complex species to antifungal pressure, highlighting the intricate interplay between resistance mechanisms and pathogenic potential.

Materials and Methods

Clinical samples

A total of 17 samples of the *C. parapsilosis* complex were evaluated, comprising 6 *C. parapsilosis* sensu stricto, 6 C. *orthopsilosis*, and 5 *C. metapsilosis*. The samples were isolated from various clinical specimens, including nails, skin (hands, feet, face, and body), urine and vaginal secretion. These samples were obtained from the fungal collection of the Hospital de Clínicas da Universidade Federal do Triângulo Mineiro (UFTM) and other medicais centers and practices in Uberaba, MG, Brazil. All samples were phenotypically identified using slide cultivation on cornmeal agar, biochemical tests (urease test, auxanography, and zymogram), and culture on CHROMagar Candida. Molecular identification was subsequently performed by sequencing the ITS region of ribosomal DNA.

Induction of Resistance and Evaluation of Stability

To assess the potential for resistance development to FLC and CSF, samples were exposed to increasing concentrations of these antifungal agents independently, following the methodology proposed by Fekete-Forgács et al., 2000. Simultaneously, a control process was conducted without the antifungal agent to confirm that any observed resistance was not due to spontaneous mutations. To evaluate resistance stability, the resistant samples were subcultured daily on Sabouraud dextrose agar (SDA) (Himedia Laboratories, Mumbai, India) without antifungal agents for up to 30 days, after which the minimal inhibitory concentration (MIC) was reassessed (Pippi et al., 2015).

***In vitro* antifungal susceptibility assay**

The MICs of FLC (Pfizer), itraconazole (ITC; Janssen Pharmaceutica), voriconazole (VRC; Pfizer), amphotericin B (AMB; Bristol-Myers-Squib), CSF (Merck Sharp & Dohme), micafungin (MCF; Astellas Pharma), and anidulafungin (ANF; Pfizer) against planktonic cells of *C. parapsilosis* complex species were determined using broth microdilution method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) documents M27-A3 (CLSI, 2008a) and M27-S4 (CLSI, 2012). The concentrations of the antifungal agents ranged from 0.03 to 8 μg/mL for ITC, VRC, AMB, CSF, MCF, and ANF, and from 0.125 to 64 μg/mL for FLC.

The MIC for AMB was defined as the lowest concentration that prevented any discernible yeast growth. For azole derivatives and echinocandins, the MICs were defined as the lowest concentration capable of inhibiting 50% of yeast growth relative to the drug-free growth control. The interpretive breakpoints for antifungal susceptibility were as follows:

- For FLC, MICs ≤ 2 μg/mL indicate susceptibility, 4 μg/mL indicate dose-dependent susceptibility, and ≥ 8 μg/mL indicate resistance (CLSI, 2012).
- For ITC and VRC, MICs ≤ 0.12 μg/mL indicate susceptibility, 0.25–0.5 μg/mL indicate dose-dependent susceptibility, and ≥ 1 μg/mL indicate resistance (CLSI, 2008b; CLSI, 2012).
- For AMB, MICs ≥ 1 μg/mL indicate decreased susceptibility (CLSI, 2008a).
- For CSF, ANF, and MCF, MICs ≤ 2 μg/mL indicate susceptibility, 4 μg/mL indicate intermediate susceptibility, and ≥ 8 μg/mL indicate resistance (CLSI, 2012).

Phospholipase Production

The isolates were evaluated for phospholipase activity by measuring the diameter of the precipitation zone on egg yolk agar, following the method of Price et al. (1982), with modifications. The test medium comprised SDA, 1 mol/L NaCl, 0.005 mol/L $CaCl_2$, and 8% sterile egg yolk emulsion (Sigma-Aldrich). Each isolate was inoculated in duplicate by applying 10 microliters of a suspension containing 10^7 yeast cells per mL, standardized using a Neubauer chamber, onto the medium's surface. The plates were incubated at 37°C for 7 days, after which the diameter of the precipitation zone surrounding each colony was measured.

Protease Production

Protease production was determined based on the method described by Aoki et al., 1990. Yeasts grown for 18-24 hours on SDA were suspended in saline solution (0.9% NaCl) to achieve a final concentration of 1 x 10^7 CFU/mL, as adjusted using a Neubauer Chamber. Five microliters of this suspension were inoculated onto the surface of a culture medium containing 0.04 g $MgSO_4.7H_2O$, 0.5 g K_2HPO_4, 1.0 g NaCl, 0.2 g yeast extract, 4.0 g glucose, and 0.5 g SDA in 60 mL distilled water. The pH of the solution was adjusted to 3.5 with 1N HCl, filtered, and then added to 140 mL of autoclaved bacteriological agar (2.0%). Plates were incubated at 36°C for 7 days.

Hemolysin Production

Hemolysin production was evaluated using the method described by Luo et al., 2001, and Manns et al., 1994. Yeasts grown for 18-24 hours on SDA were suspended in saline solution (0.9% NaCl), and the cell concentration was adjusted to 1 x 10^8 yeasts/mL using a Neubauer chamber. Ten microliters of this suspension were inoculated into culture medium containing SDA supplemented with 3.0% glucose and 7.0% defibrinated sheep blood. The plates were incubated at 37°C for 48 hours in 5% CO_2.

Esterase production

Esterase activity was determined using the opacity test medium as described by Slifkin, 2000. The culture medium, distributed in Petri dishes, was prepared with 10 g bacteriological peptone, 0.1 g $CaCl_2$, 5.0 g NaCl, 15.0 g bacteriological agar, 5 mL Tween 80-, and 1000-mL distilled water. The inoculum was prepared from yeast cultures grown

on SDA for 18-24 hours, suspended in saline solution (0.9% NaCl), and adjusted to a concentration of 1 x 10^7 CFU/mL using a Neubauer chamber. Five microliters of the yeast suspension were inoculated into the test medium, and the plates were incubated at 30°C for 10 days.

Interpretation of proteinase, phospholipase, hemolysin and esterase tests

Enzyme activities were evaluated by determining the precipitation zone (Pz). The precipitation halos (for proteases, phospholipases, and esterases) and the translucent halos (for hemolysins) were measured. The Pz was calculated as the ratio of the colony diameter (mm) to the sum of the colony diameter and the diameter of the precipitation zone (mm) Price et al., 1982. A positive test was characterized by the presence of a whitish or translucent halo around the colony, whereas a negative test showed no halo formation. Enzymatic activities were classified based on the Pz value as follows (Noumi et al., 2010).

- Pz equal to 1.0: negative activity
- Pz between 0.9-0.99: very low enzymatic activity
- Pz between 0.8-0.89: low enzymatic activity
- Pz between 0.70-0.79: high enzymatic activity
- Pz < 0.69: very high enzymatic activity

Biofilm formation

Biofilms were produced in untreated sterile 96-well polyethylene plates, according to Jin et al., 2003, with modifications. Initially, 100 µl of a standardized cell suspension (10^7cells/ml) was transferred into each well of a microtiter plate, and the plate was incubated for 1.5 h at 37°C to allow the yeast to adhere to the well surfaces. As controls, three wells of each microtiter plate were processed identically, except that no *Candida* suspensions were added.

Following the adhesion phase, the cell suspensions were aspirated, and each well was washed twice with 150 µl of saline solution (0.9% NaCl) to remove loosely adherent cells. Subsequently, 100 µl of RPMI-1640 medium buffered to pH 7.0 with MOPS was added to each washed well, and the plates were incubated at 37°C. Biofilms were allowed to develop for up to 48 h. Yeast quantification was performed using both the XTT reduction assay and the crystal violet assay (described below). The medium was replenished daily by aspirating the spent medium and adding fresh medium. All assays were conducted on three separate occasions in triplicate.

XTT reduction assay

The 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) - 5-[(phenylamino)carbonyl] - 2Htetrazolium hydroxide (XTT) (Sigma-Aldrich) reduction assay was performed as a measure of metabolic activity in order to estimate the burden of viable cells, essentially as described by Melo et al., 2011. The biofilm in each well was washed twice with 200 µL of saline solution (0.9% NaCl). The XTT-menadione solution was prepared on the test day of the assay by adding 1.5 mL of XTT (1 mg/mL in sterile saline; Sigma-Aldrich) in 300 µL of menadione solution (0.4 mM in acetone; Sigma-Aldrich). Plates were incubated for 3h in the dark at 35°C. Subsequently, 100 µL of the reaction mixture was transferred to a new microplate, and absorbance was measured at 492 nm using a BioRad ELISA reader. The biofilm formation capacity of *Candida* species was classified based on OD492 values, where OD492 ≥ 0.2 indicated positive biofilm formation and OD492 < 0.2 indicated absence of biofilm formation (de Toro et al., 2011).

Biomass quantification by Crystal Violet Staining

Biofilm biomass was quantified using a 0.4% crystal violet solution, following the method of Melo et al., 2011. Briefly, the biofilm in each well was washed twice with 200 µL of saline solution (0.9% NaCl) and air-dried for 20 minutes. Then, 110 µL of 0.4% crystal violet aqueous solution was added to each well for 45 minutes. After staining, the wells were washed three times with 200 µL of sterile Milli-Q water and immediately destained with 200 µL of 95% ethanol. After 45 minutes of destaining, the amount of crystal violet retained was measured at 595 nm using a BioRad ELISA reader. Absorbance values were corrected by subtracting the absorbance of the control wells to quantify the biofilm biomass.

***Galleria mellonella* survival assay**

To evaluate the pathogenicity of *Candida parapsilosis* complex isolates before and after exposure to FLC and CSF, two isolates each of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis* were randomly selected for analyses using the *G. mellonella* infection model. In brief, ten healthy sixth-instar *G. mellonella* larvae (weighing 0.25±0.03 g) were randomly chosen for each experimental group. The proleg area of each larva was disinfected with 70% ethanol prior to inoculation. Each larva received an injection of 10 µL of a *Candida* suspension containing 5×10^8 yeasts/mL (5

× 10^6 yeasts per larva) using a Hamilton microsyringe (Hamilton syringe 701N, volume 10 µL, needle size 26s ga - cone tip). Control groups were injected with 10 µL sterile saline. The larvae were incubated in the dark at 28°C in glass Petri dishes, and survival was assessed daily for 7 days. Larvae were considered dead if they did not move when touched with forceps. At least two independent experiments were performed for each *C. parapsilosis* complex strain tested.

Killing curves were analyzed using the Kaplan–Meier method with Graph Pad Prism 5 software. Differences in the median survival among species were evaluated using the Log-rank (Mantel-Cox) test.

Results and discussion

Following the exposure of 17 isolates of the *Candida parapsilosis* complex (6 *C. parapsilosis* sensu stricto, 6 *C. orthopsilosis* and 5 *C. metapsilosis*) to progressively increasing concentrations of FLC and CSF, we determined the Minimum Inhibitory Concentration (MIC) values of these antifungals post-exposure (postMICs) and compared them to the initial MICs (pre-exposure MICs - preMICs) (Table 1).

For FLC, the preMICs range was 0.25-32 µg/ml, while postMICs ranged from 4-64 µg/ml. Except for isolate CM48S, all isolates exhibited a reduction in FLC susceptibility. Overall, FLC postMICs were significantly elevated ($p<0.001$) compared to FLC preMICs (Table 2). This elevation in MICs ranged from 2 to 128-fold. Among the 14 initially susceptible (S) isolates, 12 transitioned to resistant (R), while 2 displayed dose-dependent susceptibility (SDD). The SDD isolate CP145 progressed to R, whereas exposure to FLC did not elevate the MIC for isolate CM 48s, one of the two pre-existing R isolates (Table 1).

Following subculturing for 15 and 30 days on FLC-free agar, a notable decrease in MICs was observed compared to postMICs ($p<0.001$), yet these MICs remained significantly higher than preMICs (Table 2). Importantly, MIC values remained unaltered for all samples between 15 and 30 days of subculturing (Table 1). Of the 11 samples that exhibited MIC reduction postsubculturing, only one displayed a phenotypic alteration (sample B8, transitioning from R to SDD). Consequently, 14 samples maintained resistance to FLZ, while the two SDD samples retained identical MIC values after 30 days of growth without the selective pressure of the antifungal agent (Table 1).

Table 1: Distribution of minimum inhibitory concentration (MIC) values of fluconazole (FLC) and caspofungin (CSF) for the *Candida parapsilosis* complex isolates before and after progressive antifungal exposure, and after subsequent culture in drug-free medium for 15 and 30 days.

Isolates	MIC (µg/ml)/phenotype			
	Pre-exposure (FLC; CAS)	Post-exposure (FLC; CAS)	Number of subcultures in drug-free medium	
			15 (FLC; CSF)	30 (FLC; CSF)
CP79*	0.25/S; 2/S	8/R; >8/R	8/R; >8/R	8/R; >8/R
CP87	2/S; 0,5/S	64/R; >8/R	8/R; >8/R	8/R; >8/R
CP145	4/SDD; 1/S	32/R; >8/R	16/R;4/I	16/R; 4/I
CP196	2/S; 1/S	32/R; >8/R	16/R; >8/R	16/R; >8/R
CP236	2/S; 1/S	16/R; >8/R	8/R; 4/I	8/R; 4/I
CP241	2/R; 0,5/S	16/R; >8/R	8/R; >8/R	8/R; >8/R
CO01S†	0,25/S; 0,25/S	32/R; >8/R	8/R; >8/R	8/R; >8R
COB3	2/S; 0,5/S	64/R; >8/R	8/R; 4/I	8/R; 4/I
CO22	2/S; 0,25/S	32/R; >8/R	16/R; >8/R	16/R; >8R
CO142	1/S; 1/S	4/SDD; >8/R	4/SDD; >8/R	4/SDD; >8R
CO226	16/R; 0,5/S	32/R; >8/R	16/R; >8/R	16/R; >8R
CO291	1/S; 0,5/S	4/SDD; >8/R	4/SDD; >8/R	4/SDD; >8R
CMB8‡	2/S; 0,25/S	32/R; >8/R	4/SDD; >8/R	4/SDD; >8/R
CM39S	2/S; 1/S	16/R; >8/R	16/R; >8/R	16/R; >8R
CM42	2/S; 0,5/S	16/R; >8/R	16/R; >8/R	16/R; >8R
CM48S	32/R; 0,5/S	32/R; >8/R	16/R; >8/R	16/R; >8R
CM86	1/S; 0,5/S	8/R; >8/R	8/R; >8/R	8/R; >8R

*CP = *Candida parapsilosis* sensu stricto, †CO = *Candida orthopsilosis*, ‡CM = *Candida metapsilosis*

Similarly to the findings with FLC, samples exposed to CSF exhibited a significant increase in postMIC values compared to preMICs ($p<0.001$) (Table 2). The preMICs range was 0.25-2 µg/ml, and all samples had postMIC > 8 µg/ml (the highest tested concentration), transitioning their phenotypes from S to R. After subculturing on CSF-free medium, a decrease in MIC values was observed in at least three samples (COB3, CP145, and CP236). These samples experienced a reduction in their MICs from > 8 µg/ml to 4 µg/ml; consequently, their phenotypes were altered from R to intermediate (I) (Table 1). It is noteworthy that for the control group, consisting of strains incubated successively without antifungals, the MIC values of both FLC and CSF remained stable throughout the study period.

Table 2: Statistical analysis of MICs of fluconazole and caspofungin for *Candida parapsilosis* complex isolates before and after progressive antifungal exposure, and following subsequent culture in drug-free medium for 15 and 30 days.

Condition	MIC (µg/ml)[1]	
	Fluconazole	Caspofungin
Pre-exposure	2 (0,25-32) **c**	0,5 (0,25-2) **b**
Post-exposure	16 (4-64) **a**	8 (8-8) **a**
Subcultures in drug-free 15 days	8 (4-16) **b**	8 (4-8) **a**
Subcultures in drug-free 30 days	8 (4-16) **b**	8 (4-8) **a**
p- Value[2]	<0,001	<0,001

[1]The minimum inhibitory concentration (MIC) values are expressed as Median (Minimum-Maximum).
[2]p-value corresponds to the significance level obtained via the Friedman test. Different letters in the column indicate results that exhibited significant differences following Dunn's multiple comparison test.

Exposure to CSF significantly altered the MIC values of all tested antifungals when compared to the MICs obtained for the original strains. The MICs of AMB ($p = 0.002$) and echinocandins (ANI, CSF, and MYC) ($p < 0.001$) were significantly higher than their corresponding MICs before exposure. In contrast, the MICs of azoles [FLC ($p = 0.035$), ITC ($p = 0.005$), and VRC ($p = 0.042$)] were significantly lower than their respective MICs before exposure to CSF (Table 3 and supplementary material, Table S1).

Following exposure to FLC, along with the emergence of resistance to FLC itself ($p < 0.001$), the isolates demonstrated decreased susceptibility to AMB ($p = 0.002$) and ITC ($p < 0.001$), while exhibiting increased susceptibility to CSF ($p = 0.016$). Additionally, a trend towards reduced MICs of VRC and increased MICs of ANI and MYC was observed, but these changes after FLC exposure were not statistically significant (Table 3).

Table 3: Statistical analysis of MICs of antifungals for *Candida parapsilosis* complex isolates before and after progressive antifungal exposure to fluconazole and caspofungin.

Pre-exposure		Post-exposure to fluconazole		Post-exposure to caspofungin	
Antifungal	MIC	MIC	p-value	MIC	p-value
Amphotericin B	1 (0.25-1)	2 (0.25-2)	**0.002**	2 (0.5-2)	**0.002**
Anidulafungin	1 (0.12-1)	1 (0.5-2)	0.109	4 (4-4)	**<0.001**
Caspofungin	0.5 (0.25-2)	0.5 (0.06-1)	**0.016**	8 (8-8)	**<0.001**
Flucanazole	2 (0.25-32)	16 (4-64)	**<0.001**	1 (0.5-32)	**0.035**
Itraconazole	0.12 (≤0.03-0.5)	2 (0.25-16)	**<0.001**	≤0.03 (≤0.03-0.12)	**0.005**
Micafungin	2 (1-2)	2 (0.5-4)	0.063	4 (4-4)	**<0.001**
Voriconazole	≤0.03 (≤0.03-0.5)	0.12 (≤0.03-0.5)	0.142	≤0.03 (≤0.03-0.12)	**0.042**

[1] Minimum inhibitory concentration (MIC) values were expressed as Median (Minimum-Maximum).
[2] The p-value indicates the level of significance obtained using the Wilcoxon test to compare the original samples (susceptible to fluconazole or caspofungin) with the samples with induced resistance, to assess cross-resistance to the tested antifungal agents.

These data collectively demonstrate that species within the *C. parapsilosis* complex not only can develop resistance to both FLC and CSF when exposed to increasing concentrations of these antifungals, but also, upon experiencing selective pressure from one antifungal and acquiring resistance to it, may develop cross-resistance to other antifungals.

Indeed, the ability of *Candida* species to develop resistance following prolonged exposure to antifungals is well-documented both *in vitro* and *in vivo*, evidencing that they are highly adaptable microorganisms (Brilhante et al., 2019; Fekete-Forgács et al., 2000; Martínez et al., 2022; Paul et al., 2020; Pinto e Silva et al., 2009; Rocha et al., 2016).

In vitro exposure to inceasing concentrations of FLC has been shown to result in the development of antifungal resistance in *C. albicans* (Fekete-Forgács et al., 2000) and *C. tropicalis* (Paul et al., 2020). The *in vivo* emergence of FLC resistance in C. albicans has also been demonstrated in an HIV-infected patient undergoing long-term FLC therapy for oropharyngeal candidiasis (Martínez et al., 2002). Likewise, FLC-resistant *C. parapsilosis* has emerged from a persistent candidemia patient with prolonged antifungal therapy (Zhang et al., 2015).

Within the *C. parapsilosis* complex, prior studies have also demonstrated laboratoryinduced antifungal resistance. Pinto e Silva et al. (2009) observed that exposure of *C. parapsilosis* strains to FLC resulted in increased MIC values not only to FLC itself but also to VRC, leading to a shift in susceptibility phenotype from S to R. Furthermore, they demonstrated that the resistance pattern remained stable after 30 days of growth in a drug-free medium. Similar results were reported by Brilhante et al. (2019), who showed that exposure of the three cryptic species of the *C. parapsilosis* complex to azoles (FLC and agricultural azoles) led to the development of resistance to the antifungals they were exposed to, and this resistance remained stable after 15 days of growth in an antifungal-free medium. Interestingly, these authors demonstrated that exposure to agricultural azoles significantly increased FLC MICs, whereas FLC exposure decreased susceptibility of *C. parapsilosi*s species to VRC. However, exposure to agricultural azoles did not alter susceptibility to VRC and ITC, and FLC exposure did not induce an increase in ITC MICs (Brilhante et al., 2019).

Conversely, Rocha et al. (2016) presented contrasting findings regarding the crossresistance profile to azoles. They observed that exposure of *C. parapsilosis* sensu stricto to tetraconazole, an agricultural azole, led to reduced susceptibility to FLC, ITC, and VRC, similar to the effects seen in the group exposed to FLC. These results

emphasize the concern that resistance to clinically used azoles might be exacerbated by the use of azoles in agriculture, in addition to the extensive clinical application of azoles, particularly FLC, for the treatment and prophylaxis of invasive candidiasis. The emergence of azole-resistant *C. parapsilosis* strains has indeed been increasing in recent years (Ning et al., 2023). As reported by Papp et al. (2020) while the development of azole resistance during invasive disease with C. albicans remains uncommon, azole-resistant *C. parapsilosis* strains are frequently isolated in hospital environments.

Collectively, our data, in conjunction with existing studies, demonstrate that members of the *C. parapsilosis* complex can develop cross-resistance to various azoles following exposure to a specific azole, whether of clinical or agricultural origin. However, the crossresistance patterns may vary. The divergent outcomes observed in different studies regarding cross-resistance patterns may be attributed to factors such as the specific antifungal agent applied, the genetic characteristics of the strains used, disparities in the methodologies employed for generating resistant strains, and the molecular mechanisms of resistance developed by these strains.

It has been documented that azole resistance in the *C. parapsilosis* complex can occur through various mechanisms, including mutation and/or overexpression of genes associated with ergosterol biosynthesis (ERG11) and genes encoding transmembrane efflux pumps (MDR1 and/or CDR1) (Brilhante et al., 2019). However, it has been demonstrated that in FLCresistant *C. parapsilosis*, overexpression of efflux pumps is more prevalent than overexpression of ERG11 (Czajka et al., 2023; Rocha et al., 2016), and this efflux pump overexpression can lead to the acquisition of azole cross-resistance in *C. parapsilosis* (Zhang et al., 2015).

While the development of azole resistance, particularly to FLC, is well-documented among *Candida* species, resistance to echinocandins has been rarely observed, although *C. glabrata* (*Nakaseomyces glabrata*) shows a greater propensity to develop resistance (Ning et al., 2023) and *C. parapsilosis* tends to exhibit higher MICs to echinocandins compared to other *Candida* species (2 mg/L vs 0.25 mg/L for *C. parapsilosis* and *C. albicans*, respectively (Pristov and Ghannoum, 2019; Zhang et al., 2015). Even with these high MIC values, patients with systemic infections respond well to echinocandin treatments, but repeated exposure to echinocandins is a risk factor for *C. parapsilosis* developing resistance (Pristov and Ghannoum, 2019).

Our study demonstrated that, similar to what occurs in *C. glabrata* (Bordallo-Cardona et al., 2017), the induction of resistance in *C. parapsilosis* species following

exposure to increasing concentrations of CSF leads to increased MIC values or even resistance to all three echinocandins. Similar results were found by Papp et al. (2018), who demonstrated that CSF resistance development in *C. parapsilosis* sensu stricto leads to resistance to other echinocandins. In line with these findings, it has been observed that strains of echinocandinresistant *Candida* typically exhibit this phenotype for all agents of this class, but they usually do not show cross-resistance to azoles (Czajka et al., 2023). Considering the inherent diminished susceptibility of *C. parapsilosis* to echinocandins, the emergence of azole resistance is undeniably a matter of concern (Díaz-García et al., 2022).

However, our study reveals for the first time, to the best of our knowledge, an inverse correlation between the MICs of azoles and echinocandins in *C. parapsilosis* strains with induced resistance to FLC and CSF (Table 3). Specifically, FLC-resistant strains exhibit elevated MICs for azoles and reduced MICs for echinocandins compared to the original isolates. Conversely, strains with induced resistance to CSF show increased MIC values for echinocandins and decreased MICs for azoles. These findings are consistent with studies demonstrating the efficacy of echinocandins as a treatment for azole-resistant *C. parapsilosis* isolates (Ning et al., 2023) and suggest that azoles may represent viable therapeutic options for infections caused by echinocandin-resistant *C. parapsilosis* species.

Further studies are required to determine if the observed inverse relationship between azole and echinocandin MICs is a common phenomenon following the development of resistance to one of these antifungal classes in *C. parapsilosis* complex species. The literature contains conflicting data, at least for *C. parapsilosis* sensu stricto samples. Strains of this species with induced CSF resistance showed slightly increased MIC values for FLC and ITC, but the MICs for VOR and posaconazole (POS) remained unchanged compared to the parental *C. parapsilosis* strain (Papp et al., 2018). Conversely, Papp et al. (2020) reported slight decreases in echinocandin MIC values in *C. parapsilosis* sensu stricto samples following the development of resistance to FLC or VOR, while resistance to posaconazole led to 2- to 4-fold increases in echinocandin MICs. Additionally, Chassot et al. (2016) observed a positive relationship between the acquisition of echinocandin resistance and increased MIC values for FLC and VOR in *C. parapsilosis* sensu stricto strains. The emergence of isolates simultaneously resistant to

both azole and echinocandin antifungals would significantly limit the available therapeutic options (Zhang et al., 2015).

A notable finding was the significant increase in AMB MICs in samples with induced resistance to both FLC and CSF. Reports of isolated fungal strains with acquired polyene resistance are relatively rare, despite decades of clinical use. This rarity may be attributed to the effectiveness of their fungicidal activity, which prevents the evolution of stable resistant mutants (Czajka et al., 2023). However, although rarely described, cross-resistance to azole and polyene drugs can occur in *Candida* species as a result of a decrease or total absence of ergosterol in the plasma membrane through mutations in ergosterol biosynthesis genes, such as ERG11 in *C. albicans* (Vandeputte et al., 2012). Similar results to ours, with AMB MIC values for FLC-resistant *C. parapsilosis* strains increased 2- to 8-fold compared to those for the parental strains, were also observed by Papp et al. (2020).

It is well-established that strains of echinocandin-resistant *Candida* usually do not exhibit cross-resistance to AMB (Czajka et al., 2023). Contrary to this, our data revealed an unexpected *in vitro* loss of susceptibility to AMB following the exposure of *C. parapsilosis* complex strains to CSF: all isolates were susceptible to AMB (MIC $\leq$ 1 µg/ml) before exposure to CSF, while 9 strains (53%) became non-susceptible after the development of resistance to CSF. Results different from ours were reported by Chassot et al. (2016), who observed that all echinocandin-resistant *C. parapsilosis* sensu stricto isolates remained susceptible to AMB. These findings underscore the complexity of antifungal resistance mechanisms in *C. parapsilosis* and highlight the need for ongoing surveillance and research to optimize treatment strategies.

In the next set of experiments, we evaluated the effects of acquiring resistance to CSF and fluconazole (FLC) on recognized virulence factors of *C. parapsilosis* complex species, including the secretion of hydrolytic enzymes (esterase, phospholipase, aspartic protease, and haemolysin) and biofilm formation.

Regarding extracellular hydrolytic activities, prior to antifungal exposure, esterase and haemolysin activities were the most common among *C. parapsilosis* complex species, with high or very high activity observed in 12/17 isolates. In contrast, high or very high protease activity was found in 5/17 isolates, and only 2/17 isolates were capable of producing phospholipases (Figure 1). After induction of resistance to FLC, the number of strains with high or very high activity for esterase, haemolysin, protease, and

phospholipase were 6, 12, 7, and 0, respectively. Following CSF resistance induction, the numbers were 8, 10, 6, and 0, respectively. No statistically significant differences were observed in exoenzyme activity between non-induced samples and those with induced resistance to FLC or CSF (supplementary material, Tables S2 and S3).

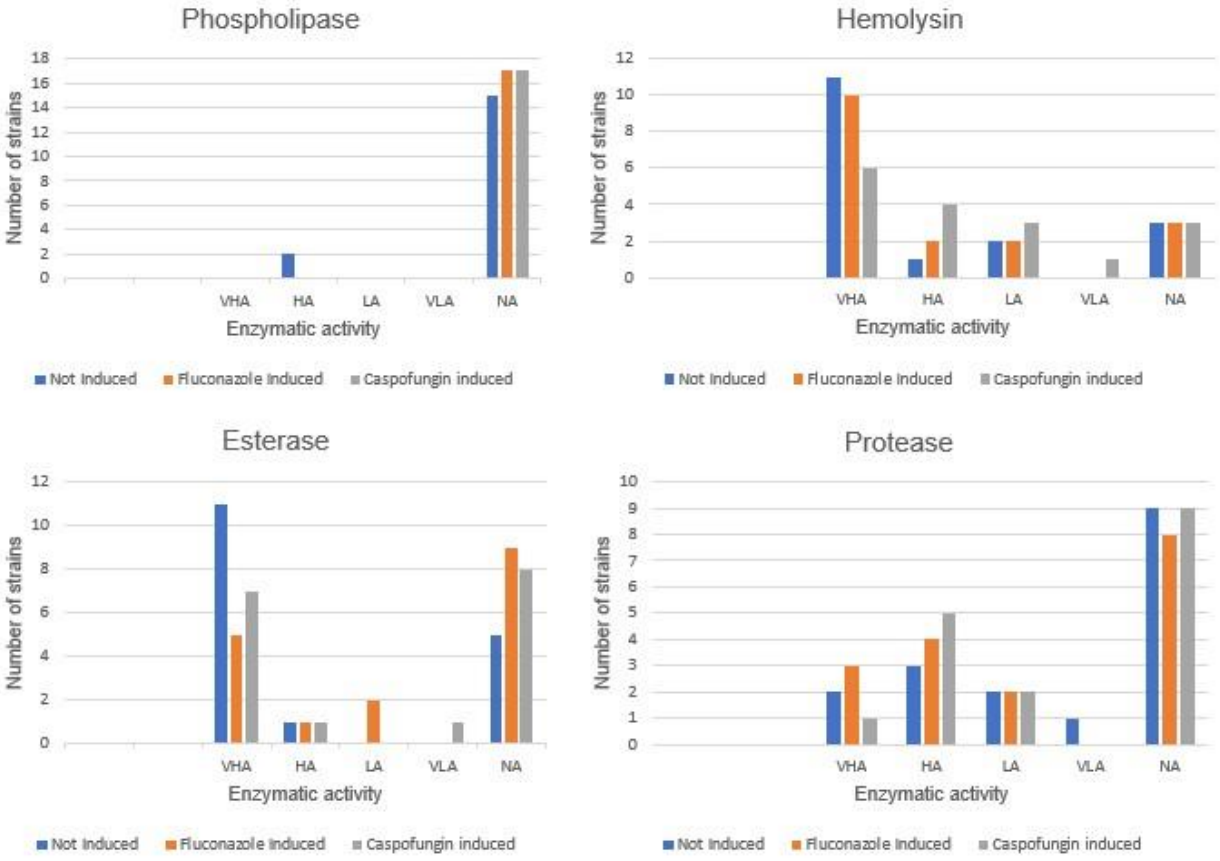

Figure 1: Production of hydrolytic enzymes in *C. parapsilosis* complex species before and after progressive exposure to fluconazole and caspofungin. NA: negative activity, VLA: very low enzymatic activity, LA: low enzymatic activity, HA: high enzymatic activity, VHA: very high enzymatic activity.

Secretion of hydrolytic enzymes is thought to play an important role in the pathogenesis of disease caused by *C. parapsilosis*, facilitating its adherence and tissue invasion, damaging host immune cells to avoid antimicrobial attack, degrading immunological defense proteins such as antibodies and complement, and enabling the acquisition of iron and nutrients from the host (Németh et al., 2013). The literature indicates that *C. parapsilosis* complex isolates produce esterases, phospholipases, proteases, and haemolysins (Brilhante et al., 2018; TreviñoRangel et al., 2013), and our study corroborates these findings. However, there have been contradictory results

regarding the level of activity of these enzymes within the members of the *C. parapsilosis* complex. For example, in a study conducted by Treviño-Rangel et al 2013, only 17% of the total isolates tested demonstrated protease activity, making it the least produced enzyme by the *C. parapsilosis* species complex among the enzymes evaluated. Conversely, phospholipases were the most produced, being positive in 80% of the isolates. A study on the virulence attributes of animal-derived *C. parapsilosis* complex isolates reported 82% proteaseproducing strains, but none produced phospholipases (Brilhante et al., 2013). Therefore, our data reinforce the idea of a high heterogeneity in the enzyme production capacity among *C. parapsilosis* complex strains.

Regarding biofilm production, a significant increase in the biofilm-forming capacity of *C. parapsilosis* complex strains following the development of resistance to both FLC and CSF was evident, regardless of the detection method used (crystal violet biomass (Tables 4 and 5) or metabolic measurements based on XTT activity (Table 6)). No strain exhibited a reduced biofilm production capacity compared to the parental *C. parapsilosis* strains. Biomass detection revealed that after FLC exposure, only one strain remained a moderate biofilm producer, while 6 moderate producers, 1 weak producer, and 1 non-producer became strong biofilm producers. Eight strains that were strong producers retained this condition (Table 4). After CSF resistance development, in addition to the 8 strains that were already strong producers, the non-producer (1), weak producer (1), and moderate producers (7) became strong biofilm producers (Table 5).

Table 4: Biofilm biomass in *C. parapsilosis* complex species before and after progressive exposure to fluconazole.

Biofilm-forming ability				
Pre-exposure	Pos-exposure Fluconazole			
	Negative	Weak	Moderate	Strong
Negative	0	0	0	1
Weak	0	0	0	1
Moderate	0	0	1	6
Strong	0	0	0	8

Number of samples with reduced biofilm-forming ability: 0/17 (0.0%) Number of samples with unchanged biofilm-forming ability: 9/17 (52.9%).
Number of samples with increased biofilm-forming ability: 8/17 (47.1%).
McNemar-Bowker test: p=0.046

Table 5: Biofilm biomass in *C. parapsilosis* complex species before and after progressive exposure to caspofungin.

Biofilm-forming ability				
Pre-exposure	Pos-exposure Caspofungin			
	Negative	Weak	Moderate	Strong
Negative	0	0	0	1
Weak	0	0	0	1
Moderate	0	0	0	7
Strong	0	0	0	8

Number of samples with reduced biofilm-forming ability: 0/17 (0.0%)
Number of samples with unchanged biofilm-forming ability: 8/17 (47.1%)
Number of samples with increased biofilm-forming ability: 9/17 (52.9%)
McNemar-Bowker test: p=0.029

In terms of metabolic activity of biofilms, the induction of resistance to FLC and CSF resulted in similar effects on the *C. parapsilosis* complex strains: 11 strains that were already capable of forming biofilms maintained this ability, while 6 non-producing isolates gained the capacity to form biofilms (Table 6). Previous studies reviewed by Bohner et al., 2022 have also demonstrated a positive correlation between increased FLC MICs and the biofilm-forming ability of *Candida* species. Possible molecular mechanisms connecting the acquisition of antifungal resistance to altered pathogenic potential in *Candida* spp. have been suggested, such as mutations in transcription factors responsible for distinct functions. These mutations could impact genes responsible for biofilm formation as well as the expression of efflux pumps (Bohner et al., 2022).

Table 6: Biofilm metabolism in *C. parapsilosis* complex species before and after progressive exposure to fluconazole (FLC) and caspofungin (CSF).

Biofilm-forming ability		
Pre-exposure strains	Pos-exposure (FLC or CSF)	
	Negative	Positive
Negative	0	6
Positive	0	11

Number of samples with unchanged biofilm-forming ability: 11/17 (64.7%)
Number of samples with increased biofilm-forming ability: 6/17 (35.3%)
Teste de McNemar: p=0.031

The increased capacity to form biofilms following the development of resistance to FLC and CSF is concerning. *C. parapsilosis* complex-related infections pose a serious global health threat, in part due to the ability to form complex biofilms, particularly on central venous catheters and other medical implants. This biofilm formation leads to significant challenges in disease management (Lass-Flörl et al., 2024).

Next, we compared the *in vivo* virulence of *C. parapsilosis* complex species before and after exposure to FLC and CSF using the invertebrate model *Galleria mellonella*. In all experiments, 10 larvae were injected with 5×10^6 yeast cells, and their survival was monitored for 7 days. In total, two isolates each (randomly chosen) of *C. parapsilosis* sensu stricto (CP241, CP236), *C. orthopsilosis* (COB3, CO142), and *C. metapsilosis* (CM39S, CM86) were used. Our results show that the mortality rate of a composite of all *C. metapsilosis*-infected larvae was significantly lower than those infected with *C. parapsilosis* sensu stricto or *C. orthopsilosis* strains in both the pre-exposure group and the CSF-resistant group (Figure 2). However, there was no significant difference in mortality rates caused by the three species in the group infected with FLC-resistant strains. In none of the groups was there a significant difference between the survival curves of larvae infected with *C. parapsilosis* sensu stricto and *C. orthopsilosis* isolates. These data indicate that *Candida metapsilosis* is the least virulent, with a median survival longer than that observed with the other two species, consistent with previous studies evaluating the virulence of the species complex in the *G. mellonella* model (Gago et al., 2014; Németh et al., 2013).

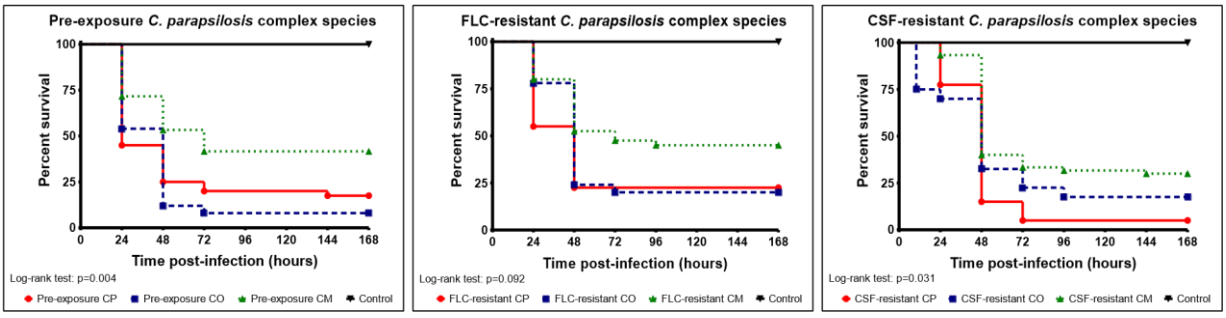

Figure 2: Survival of *Galleria mellonella* larvae infected with isolates of *C. parapsilosis* complex species before and after induced resistance to fluconazole (FLC) and caspofungin (CSF). Summarized survival curves of *G. mellonella* infected with two different strains of *C. parapsilosis* sensu stricto (CP), *C. orthopsilosis* (CO), and *C. metapsilosis* (CM) at a final concentration of 5×10^6 yeasts/larva, incubated at 28ºC.

We also assessed the impact of antifungal resistance acquisition on the virulence of each of the three species in the complex. To do this, we compared the survival curves of larvae infected with pre-exposure strains to those infected with post-exposure strains resistant to FLC and CSF (Figure 3). For none of the species was there a significant difference between the survival curves of the different groups, indicating that the acquisition of antifungal resistance did not alter the *in vivo* virulence of *C. parapsilosis* complex species, at least in the *G. mellonella* model. Our data partially corroborate *in vivo* findings in a mouse model of systemic candidiasis, which demonstrated that a *C. parapsilosis* sensu stricto strain resistant to FLC showed no virulence attenuation (Papp et al., 2020). Conversely, our results diverge from another study that showed *C. parapsilosis* sensu stricto strains with acquired resistance to echinocandins (including CSF, MCF, and ANF) had attenuated virulence in *G. mellonella* compared to the susceptible wild-type strain (Papp et al., 2018).

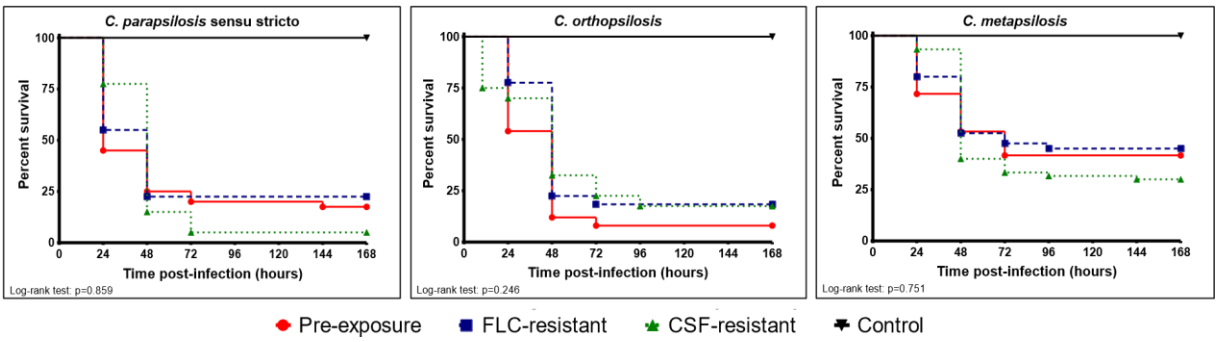

Figure 3: Survival of *Galleria mellonella* larvae infected with isolates of *C. parapsilosis* complex species before and after induced resistance to fluconazole (FLC) and caspofungin (CSF). Summarized survival proportions of *G. mellonella* infected with two different strains each of *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*, both pre-exposure (susceptible) and post-exposure (resistant) isolates to FLC and CSF. Larvae were infected at a final concentration of 5×10^6 yeasts/larva and incubated at 28ºC.

In summary, this study provides significant insights into the adaptive responses of *C. parapsilosis* complex species under antifungal pressure, underscoring the necessity for continuous surveillance and the development of alternative therapeutic strategies to manage infections by these increasingly resistant pathogens. Further research is essential to elucidate the molecular mechanisms underlying the observed resistance and cross-resistance patterns, and to explore potential strategies to counteract these adaptations.

Author contributions: CRediT

B.V.S.: Investigation, Visualization, Writing – original draft, Writing – review and editing. **D.B.C.O.**: Investigation. **I.T.J.F.**: Investigation. **R.T.O.**: Investigation. **R.O.B.**: Investigation. **G.A.N.N.**: Formal analysis, Visualization, Writing – review and editing. **A.A.A.**:

Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review and editing.

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Competing Interests:

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

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Supplementary material

Table S1: Distribution of minimum inhibitory concentration (MIC) values of antifungals for *candida parapsilosis* complex isolates before (Pre-exposure) and after (FLC-R; CSF-R) progressive exposure to fluconazole (FLC) and caspofungin (CSF).

Isolates	MIC (µg/ml)						
	Pre-exposure (FLC-R; CSF-R)						
	AMB	ANF	CSF	MCF	FLC	ITC	VRC
CP79*	0.5 (2; 1)	0.5 (0.5; 4)	2 (0.06; >8)	2 (2; 4)	0.25 (8; 1)	0.06 (16; ≤0.03)	0.25 (0.06; ≤0.03)
CP87	0.5 (1; 0.5)	1 (2; 4)	0.5 (0.5; >8)	2 (2; 4)	2 (64; 0.5)	≤0.03 (2; ≤0.03)	≤0.03 (0.25; ≤0.03)
CP145	0.25 (1; 2)	1 (1; 4)	1 (0.5; >8)	2 (4; 4)	4 (32; 1)	0.12 (0.5; 0,.12)	≤0.03 (0.25; ≤0.03)
CP196	0.5 (1; 2)	1 (2; 4)	1 (1; >8)	2 (2; 4)	2 (32; 1)	0.5 (1; ≤0.03)	0.06 (0.25; ≤0.03)
CP236	1 (2; 1)	1 (1; 4)	1 (0.25; >8)	2 (4; 4)	2 (16; 1)	0.12 (1; ≤0.03)	≤0.03 (≤0.03; ≤0.03)
CP241	0.25 (2; 0.5)	0.5 (1; 4)	0.5 (0.5; >8)	2 (2; 4)	2 (16; 0.5)	0.25 (4; ≤0.03)	≤0.03 (0.25; ≤0.03)
CO01S†	1 (0.25; 1)	0.5 (1; 4)	0.25 (0.5; >8)	1 (2; 4)	0.25 (32; 1)	0.25 (0.5; ≤0.03)	≤0.03 (0.25; ≤0.03)
COB3	1 (2; 2)	0.5 (0.5; 4)	0.5 (0.25; >8)	1 (1; 4)	2 (64; 2)	0.12 (2; 0.12)	0.12 (0.5; 0.12)
CO22	1 (1; 2)	1 (0.5; 4)	0.25 (0.5; >8)	2 (2; 4)	2 (32; 2)	≤0.03 (1; ≤0.03)	≤0.03 (0.25; ≤0.03)
CO142	1 (0.25; 1)	1 (1; 4)	1 (0.25; >8)	2 (1; 4)	2 (4; 1)	0.25 (0.25; 0.06)	≤0.03 (≤0.03; 0.06)
CO226	1 (2; 1)	1 (0.5; 4)	0.5 (0.5; >8)	1 (0.5; 4)	16 (32; 32)	0.12 (2; 0.12)	0.12 (0.25; ≤0.03)
CO291	1 (2; 2)	0.5 (1; 4)	0.5 (0.12; >8)	1 (1; 4)	1 (4; 1)	≤0.03 (4; ≤0.03)	≤0.03 (≤0.03; ≤0.03)
CMB8‡	1 (2; 2)	0.12 (1; 4)	0.25 (0.12; >8)	1 (2; 4)	2 (32; 2)	0.12 (2; ≤0.03)	0.06 (0.12; ≤0.03)
CM39S	1 (2; 2)	0.5 (1; 4)	1 (0.12; >8)	1 (2; 4)	2 (16; 2)	0.25 (1; ≤0.03)	0.12 (0.06; ≤0.03)
CM42	1 (2; 2)	1 (1; 4)	0.5 (0.5; >8)	2 (2; 4)	2 (16; 2)	0.25 (2; ≤0.03)	≤0.03 (0.12; ≤0.03)
CM48S	1 (2; 2)	0.25 (0.5; 4)	0.5 (0.5; >8)	1 (1; 4)	32 (32; 32)	0.12 (2; 0.12)	0.5 (0.25; ≤0.03)
CM86	0.5 (2; 1)	1 (0.5; 4)	0.5 (0.06; >8)	1 (1; 4)	1 (8; 1)	0.06 (1; ≤0.03)	≤0.03 (≤0.03; ≤0.03)

*CP = *Candida parapsilosis* sensu stricto, †CO = *Candida orthopsilosis*, ‡CM = *Candida metapsilosis*

AMB - amphotericin B, ANF - anidulafungin, CSF - caspofungin, MCF - micafungin, FLC - fluconazole, ITC - itraconazole, VRC - voriconazole.

Table S2: Statistical analysis of production of hydrolytic enzymes for *Candida parapsilosis* complex isolates before and after progressive antifungal exposure to fluconazole.

Production of hydrolytic enzymes				
Pre-exposure	Post-exposure to fluconazole			p-value*
	Decreased	Unchanged	Increased	
Esterase	7/17 (41.2%)	9/17 (52.9%)	1/17 (5.9%)	0.156
Phospholipase	2/17 (11.8%)	15/17 (88.2%)	0/17 (0.0%)	0.157
Haemolysin	6/17 (35.3%)	7/17 (41.2%)	4/17 (23.5%)	0.549
Protease	4/17 (23.5%)	8/17 (47.1%)	5/17 (29.4%)	0.801

*p-value pertains to the outcome of the McNemar-Bowker test, which compares the exoenzyme activity between non-induced samples and those subjected to fluconazole resistance induction.

Table S3: Statistical analysis of production of hydrolytic enzymes for *Candida parapsilosis* complex isolates before and after progressive antifungal exposure to caspofungin.

Production of hydrolytic enzymes				
Pre-exposure	Post-exposure to caspofungin			p-value*
	Decreased	Unchanged	Increased	
Esterase	8/17 (47.1%)	7/17 (41.2%)	2/17 (11.8%)	0.225
Phospholipase	2/17 (11.8%)	15/17 (88.2%)	0/17 (0.0%)	0.157
Haemolysin	8/17 (47.1%)	4/17 (23.5%)	5/17 (29.4%)	0.518
Protease	5/17 (29.4%)	6/17 (35.3%)	6/17 (35.3%)	0.700

* p-value pertains to the outcome of the McNemar-Bowker test, which compares the exoenzyme activity between non-induced samples and those subjected to caspofungin resistance induction.

3 COMPROVANTE DE SUBMISSÃO

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4 PARTICIPAÇÃO DOS CO-AUTORES

Em atendimento ao regulamento do PPGCF, listamos abaixo a participação de cada co-autor.

Beatriz Virgínia da Silva: ensaios biológicos, figuras, redação e revisão do artigo.

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