PATRÍCIA CANTERI DE SOUZA

MODELOS INVERTEBRADOS NO ESTUDO DE INFECÇÕES FÚNGICAS E NA AVALIAÇÃO DE ANTIFÚNGICOS

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Orientador: Ricardo Sérgio Couto de Almeida

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Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

de Souza, Patricia.
Modelos Invertebrados no Estudo de Infecções Fúngicas e na Avaliação de Antifúngicos / Patricia de Souza. - Londrina, 2016.
91 f. : il.

Orientador: Ricardo Sérgio de Almeida.
Dissertação (Mestrado em Microbiologia) - Universidade Estadual de Londrina, Centro de Ciências Biológicas, Programa de Pós-Graduação em Microbiologia, 2016.
Inclui bibliografia.

1. Modelos Alternativos - Teses. 2. Antifúngicos - Teses. 3. Candida sp. - Teses. 4. Cryptococcus sp. - Teses. I. de Almeida, Ricardo Sérgio. II. Universidade Estadual de Londrina. Centro de Ciências Biológicas. Programa de Pós-Graduação em Microbiologia. III. Título.

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Londrina, 23 de Março de 2016.

Dedico este trabalho à minha querida mãe, meu amor maior, que faz uma falta inestimável e que deixou bons ensinamentos.

AGRADECIMENTOS

Agradeço primeiramente aos meus pais, que são o meu alicerce, sempre me deram todo o amor e carinho. Meu pai Aristides por ser o melhor pai, ter um bom coração e me dar força nos momentos difíceis. Minha mãe Elza por ser a pessoa que mais lutou para me dar educação, que era minha companheira e que estará sempre no meu coração;

Agradeço ao meu orientador, Ricardo Sérgio Couto de Almeida, que me acolheu como sua orientanda e me auxiliou, sempre tendo muita ambição e idéias inovadoras; ao professor Luciano Aparecido Panagio, que me orientou no início deste trabalho, na confecção da minha monografia. Foi ele que com muita paciência me ensinou os primeiros passos;

Ao professor Gerson Nakazato e ao professor Alexandre Tadachi Morey que me auxiliaram na confecção de artigos e nas escolhas das metodologias, entre outros detalhes;

A professora Márcia Cristina Furlaneto e a Terezinha Inez Estivalet Svidzinski que com toda a disposição compuseram minha banca e fizeram críticas cruciais para o meu crescimento como pesquisadora;

Ao meu noivo Gaspar, meu melhor amigo, por tornar meu mundo mais leve e bonito. Por ser essa pessoa tão dedicada e centrada, por fornecer meu apoio psicológico e por ser o meu futuro. Agradeço também aos meus sogros, Hélia e João Bosco, os melhores que eu poderia ter, por serem exemplos de vida, e também por me auxiliaram na confecção da dissertação;

A cada membro da minha família: minhas avós Camilla e Terezinha, meus tios Eloísa, Marcelo, Édna, Ivo, Zilda, Carlos e meus primos, que sempre acompanharam ativamente minhas conquistas e me ajudaram, cada um da sua forma. Agradeço em especial aos tios João Mauro e Regina que foram essenciais ao meu amadurecimento, sempre me deram bons exemplos e auxiliaram nossa família;

Aos meus colegas e amigos de laboratório, Felipe, Caísa, Tatiany, Gabriela, César, Guilherme, Ricardo e Loana que com muito bom humor tornam o trabalho mais fácil e os dias mais alegres;

Agradeço em especial às minhas amigas Karla e Aline. A Karla por ter sido uma amiga muito carinhosa e compreensiva principalmente quando mais precisei. A Aline por ser meu braço direito no laboratório, por me proporcionar conversas maduras, também a considero muito;

Aos meus amigos da graduação e do ensino médio. Já passamos por tantos momentos bons juntos, tantas recordações que foram construindo o que somos. Eu sei que essa amizade perdura e não acabará;

À Universidade Estadual de Londrina, por possibilitar a realização da minha graduação e do mestrado, ao CNPq por financiar o projeto e a CAPES por subsidiar minha bolsa de estudo;

“A felicidade do corpo consiste na saúde,
e a do espírito, na sabedoria”

(Tales de Mileto)

SOUZA, Patrícia Canteri de. **Modelos invertebrados no estudo de infecções fúngicas e na avaliação de antifúngicos.** 2016. 91 f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

É crescente o número de infecções causadas por fungos, dentre eles, o gênero *Candida* é o mais frequente e *Cryptococcus* sp. ocupa o segundo lugar. Essas leveduras causam graves doenças em pacientes imunossuprimidos e são responsáveis por alta letalidade. Diante desses fatos, a procura por novos fármacos é importante. A sinvastatina é uma droga antes conhecida por reduzir os níveis de colesterol do sangue, porém muitos artigos relatam que ela possui ação antifúngica *in vitro*, contudo, não há trabalhos *in vivo*. As larvas de *Galleria mellonella*, um modelo alternativo invertebrado já bem estabelecido, são uma opção relevante para esse estudo. Outros modelos precisam ser padronizados para serem utilizados na elucidação das infecções fúngicas. Os objetivos desse estudo foram testar a atividade de sinvastatina *in vitro* em interação com fluconazol contra espécies raras de *Candida*, testar a atividade de sinvastatina em *G. mellonella* contra *Candida albicans* e padronizar autilização de *Tenebrio molitor* como hospedeiro em infecções fúngicas. Foram feitos testes de antibiograma e microdiluição em caldo. *C. guilliermondii* apresentou a concentração inibitória mínima para sinvastatina de 312,5 μg/mL que foi dez vezes maior do que para *C. lusitaniae*. Entretanto, a interação das drogas para *C. guilliermondii* foi sinérgica, enquanto que para *C. lusitaniae* foi aditiva. A taxa de mortalidade das larvas tratadas com sinvastatina e infectadas com *C. albicans* foi alta, estatisticamente igual à taxa de mortalidade do grupo controle, que apenas recebeu o inóculo com a levedura. Obtivemos resultados semelhantes na terapia conjunta das drogas. Os testes com *T. molitor* mostraram que com o aumento da concentração dos inóculos de ambos os fungos (*C. albicans e Cryptococcus neoformans*) houve um aumento nas taxas de morte e foi possível observar a invasão nos tecidos. Assim pudemos concluir que a sinvastatina possui atividade antifúngica *in vitro*, porém não há correspondência *in vivo*. Além disso, *T. molitor* foi apresentado aqui como um novo modelo invertebrado para o estudo de infecções fúngicas.

Palavras-chaves: *Galleria mellonella*. *Tenebrio molitor*. Sinvastatina. Fluconazol. Sinergismo.

SOUZA, Patrícia Canteri de. **Invertebrate models in the study of fungal infections and evaluation of antifungal agents.** 2016. 91 p. Dissertation (Master Science in Microbiology) – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

An increasing number of infections caused by fungi, among them, *Candida* genus is the most common and *Cryptococcus* sp. is the second. This yeasts cause serious disease in immunosuppressed patients, and they are responsible for high lethality. Given these facts, the search for new drugs is important. Simvastatin is a drug before known to reduce blood cholesterol levels, but many articles report that it has action antifungal *in vitro*, however, there is no work *in vivo*. The larvae of *Galleria mellonella*, an alternative invertebrate model already well established, are a relevant option for this study. Other models need to be standardized for use in the elucidation of fungal infections. The objetives of this study were to test *in vitro* simvastatin activity in interaction with fluconazole against rare species of *Candida*, and test the activity of simvastatin in *G. mellonella* against *Candida albicans* and standardize the use of *T. molitor* as host for fungal infections. Antibiogram and microdilution in broth tests were performed. *C. guilliermondii* showed a minimum inhibitory concentration for simvastatin at 312.5 μg/mL, which was ten times greater than that for *C. lusitaniae*. However, the interaction of drugs for *C. guilliermondii* was synergistic, whereas for *C. lusitaniae* was additive. The death rate of larvae treated with simvastatin and infected with *C. albicans* was high, statistically equal to mortality of the control group that only received the inoculum with the yeast. We obtained similar results with conjoint therapy of the drugs. Tests with *T. molitor* showed that with increasing concentration of the inoculum of the both fungi (*C. albicans* and *Cryptococcus neoformans*) there was an increase in death rate and it was observed invasion in the tissues. So we could conclude that simvastatin has *in vitro* antifungal activity, although there is no correspondence *in vivo*. Furthermore, *T. molitor* was presented here as a new invertebrate model for the study of fungal infections.

Keywords: *Galleria mellonella*. *Tenebrio molitor*. Simvastatin. Fluconazole. Synergism.

LISTA DE SIGLAS E ABREVIATURAS

HMG-CoA	Hidroxi-3-methyl-glutaril-Coenzima A
OMG	Organização Mundial da Saúde
h	Hora
mg	Miligrama
%	Porcentagem
°C	Graus Celsius
LDL	*Low Density Lipoprotein*
EUCAST	*European Committee on Antimicrobial Susceptibility Testing*
CIM	Concentração Inibitória Mínima
SIDA	Síndrome da Imunodeficiência Adquirida
ANVISA	Agência Nacional de Vigilância Sanitária
ABNT	Associação Brasileira de Normas Técnicas
UTI	Unidade de Tratamento Intensivo

SUMÁRIO

1. INTRODUÇÃO

As leveduras são os principais fungos isolados de infecções disseminadas. Dentre os gêneros encontrados, *Candida* é o mais isolado, sendo *Cryptococcus* sp. o segundo mais frequênte (MAGALHÃES *et al*., 2015; PFALLER *et al*., 2015; TEIXEIRA-LOYOLA *et al*., 2013; WISPLINGHOFF *et al*., 2014). Esses gêneros são compostos por fungos oportunistas. *Candida* sp provoca micoses cutâneas e nas mucosas em pessoas saudáveis com desequilíbrio do sistema imunológico, e pode causar infecções na corrente sanguínea (candidemia) e sistêmicas (candidose) em imunussuprimidos (MEDEIROS *et al.*, 2014; SOUZA; PAULA; SOUTO, 2014). *Cryptococcus* sp. é responsável por causar criptococose nos pulmões e pode se disseminar, instalando-se em maior quantidade no cérebro, causando meningite em pessoas saudáveis e principalmente em imunussuprimidos (ALANIO; DESNOS-OLLIVER; DROMER, 2011; KIM *et al*., 2012).

A letalidade dos pacientes acometidos por candidemia e meningite criptococócita chega a ser maior que 50% (HOFFMANN-SANTOS *et al*., 2013; MARTINS *et al*., 2011; MEZZARI *et al*. 2013; WILLE *et al.*, 2013). A espécie de *Cryptococcus* sp. mais comum é *Cryptococcus neoformans* (ALANIO; DESNOS-OLLIVER; DROMER 2011; MEZZARI *et al*. 2013). A espécie de *Candida* sp. mais freqüentemente isolada em casos de candidemia de todas as regiões do mundo é *Candida albicans* (CAGGIANO *et al*., 2013; CLEVERLAND *et al*., 2015; KIM; SUDBERY, 2011; NUCCI *et al*., 2013; KREUSCH; KARSTAEDT, 2013; SANGUINETTI; POSTERARO; LASS-FLÖR, 2015; TAM *et al*., 2015). Contudo, outras espécies desse gênero consideradas incomuns, como *Candida guilliermondii* e *Candida lusitaniae* ganham importância por estarem associadas a casos de candidemia persistentes (ASNER *et al*., 2015; GAMALETSOU *et al*., 2014; JUNG *et al*., 2015).

O tratamento de infecções causadas por fungos oportunistas é muitas vezes complicado e o número de agentes antifúngicos disponíveis é limitado (NYILASI *et al*., 2010). Além disso, a eficácia sub-ótima de agentes antifúngicos *in vivo* e o aumento das taxas de resistência fúngica enfatiza a necessidade de descobrir novos fármacos e elaborar outras estratégias terapêuticas, como a combinação de diferentes classes de antifúngicos (LIONAKIS, 2011).

Uma droga que tem sido estudada é a sinvastatina, um medicamento que reduz os níveis de colesterol de lipoproteína de baixa densidade no sangue (LPS) (VILIMANOVIC

et al., 2015). As estatinas agem através da inibição da enzima HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) redutase, que atua na biossíntese do mevalonato precursor de esteróides (VILIMANOVICH *et al.*, 2015). Dessa forma, acontece a diminuição dos níveis de ergosterol de membrana dos fungos, que em última instância afeta a propagação desses (CABRAL; FIGUEROA; FARIÑA, 2013).

Alguns estudos mostraram a ação da sinvastatina contra leveduras e a sua atividade em co-administração com outros antifúngicos e comprovaram a existência de sinergia entre sinvastatina e fluconazol para as espécies mais comuns de *Candida* (BRILHANTE *et al.*, 2015; CABRAL; FIGUEROA; FARIÑA, 2013; MENEZES *et al*., 2012; NYILASI *et al.*, 2010). Os azóis são antifúngicos disponíveis no mercado que atuam inibindo a enzima 14α-esterol desmetilase, também responsável pela biossíntese do ergosterol (ARMSTRONG; TAYLOR, 2009).

A atividade antifúngica de sinvastatina ainda não foi testada *in vivo*. Uma opção relevante é o uso de modelos alternativos para tal finalidade. *Galleria mellonella* é uma mariposa, cujas larvas vivem em colméias (GALLO *et al*., 2002) e já tem sido utilizada na pesquisa de doenças infecciosas (DESALERMOS; FUCHS; MYLONAKIS, 2012). Possui grandes vantagens ética, logística e econômica sobre os modelos de mamíferos (LI *et al*, 2013) porque dispensa a aprovação pelo comitê de bioética, a criação é mais acessível e feita em larga escala. Também pode ser mantida a 37 °C, o que a torna adequada para o estudo de patógenos à temperatura do corpo humano (LI *et al*., 2013). É uma vantagem em relação a outros modelos invertebrados que não toleram essa temperatura (DESALERMOS; FUCHS; MYLONAKIS, 2012).

É importante também estudar a infecção por fungos em outros modelos nos quais ainda não foram padronizados. Um modelo alternativo em ascensão é *Tenebrio molitor* (Coleoptera), um besouro que é considerado praga de grãos armazenados (SCHROECKENSTEIN; MEIERDAVIS; BUSH, 1990), cujas larvas são utilizadas na alimentação de animais de estimação (BARKER; FITZPATRICK; DIERENFELD, 1998). *T. molitor* possui as mesmas vantagens que *G. mellonella*, contudo, é comercializado no Brasil e mais fácil de cultivar em laboratório. Esse besouro já tem sido utilizado no estudo de infecções com bactérias (DORLING; MORAES; ROLFF, 2015; TINDWA *et al.*, 2013). O único relato do uso desse invertebrado como modelo para fungos patogênicos humanos foi desenvolvimendo nessa dissertação e publicado pelo nosso grupo de pesquisa, sendo que mais estudos subsequentes devem ser realizados, visto que esse modelo se mostrou promissor.

2. REFERENCIAL BIBLIOGRÁFICO

2.1. Epidemologia das principais leveduras de importância clínica.

A maioria dos microrganismos isolados de infecções sistêmicas consistem em bactérias. Uma porção menor é constituída por leveduras e dentre essas o gênero *Candida* é o que mais prevalece, sendo o gênero *Cryptococcus* o segundo mais frequente (MAGALHÃES *et al*., 2015; PFALLER *et al*., 2015; TEIXEIRA-LOYOLA *et al*., 2013; WISPLINGHOFF *et al*., 2014). Magalhães *et al.* (2015) realizaram uma pesquisa epidemiológica no nordeste do Brasil, onde isolaram 2004 microrganismos de amostras de sangue, urina e secreções e constataram que 94,6% desses eram bactérias e 5.4% eram leveduras. Dentre esses fungos, 89.8% eram espécies do gênero *Candida* e 4.6% do gênero *Cryptococcus.* Entre pacientes com SIDA, o patógeno mais comum continua sendo *Candida* sp., e *Cryptococus* sp. é o segundo mais frequênte (GNANANKALI; SUMANA, 2014).

2.1.1. *Candida* sp.

As leveduras do gênero *Candida* são agrupadas no filo Ascomycota e apresentam ocorrência mundial (VALLABHANENI *et al.*, 2015). São encontradas na natureza em vários substratos, como em vegetais e no solo, atuando como saprófitas (MARTINS; MELO; HEINS-VACCARI, 2005). Também possuem seu habitat no corpo humano, onde são comensais podendo estar presentes na microbiota da pele, da mucosa oral, da faringe, do trato gastrointestinal e genital (GALARZA, 2009).

Em condições favoráveis podem se multiplicar intensamente e expressar seus fatores de virulência ocasionando doenças oportunistas. Estas condições estão relacionadas com alterações no sistema imunológico do hospedeiro por imunossupressão; comprometimento das barreiras físicas por queimaduras e procedimentos invasivos; e desequilíbrio da microbiota endógena pelo uso de antibióticos de amplo espectro de ação (COLOMBO *et al.*, 2013).

Quando acontece esse desequilíbrio da microbiota ou o rompimento das barreiras anatômicas do hospedeiro, esse fungo pode invadir superfícies cutâneas e mucosas, causando as candidíases oral, vaginal e cutânea (ARAÚJO PAULO MEDEIROS *et al.*, 2014; SOUZA; PAULA; SOUTO, 2014). Em pacientes imunodeprimidos, esse gênero é responsável por causar infecção na corrente sanguínea denominada candidemia. A candidemia

ao evoluir pode espalhar-se nos vários órgãos do corpo, levando à chamada candidose disseminada (GOW *et al*., 2012; KIM; SUDBERY, 2011).

Entre os fatores de risco, aparece com maior frequência o internamento em unidades de tratamento intensivo (UTI), câncer, cirurgia, uso de corticosteróides, utilização de cateter, nutrição parenteral e antibióticoterapia de longa duração (DA COSTA *et al.*, 2014; HOFFMANN-SANTO *et al*., 2013; PONGRÁCZ *et al*., 2015; WILLE *et al*., 2013). Muitos estudos relatam que a candidemia acomete principalmente indivíduos adultos e do sexo masculino (DA COSTA *et al*., 2014; MENEZES *et al*., 2015; WILLE *et al*., 2013).

A letalidade da candidemia no Brasil é alta, por volta de 50% e está aumentando conforme os anos, assim como o tempo de internação e a idade dos pacientes (HOFFMANN-SANTOS *et al*., 2013; WILLE *et al*., 2013). Wille *et al*. (2013) realizaram uma pesquisa epidemiológica na região sudeste do país, entre os anos de 1994-1999 e 2000-2004. No primeiro período, letalidade foi 53,9%, no segundo período subiu para 56,5%. O tempo médio de internação antes era de 15 dias e depois passou a ser de 19 dias e a idade média dos pacientes aumentou de 27,2 anos para 36,5 anos.

Existem aproximadamente 200 espécies diferentes de *Candida*, entretanto somente algumas são prejudiciais para a saúde humana. As espécies epidemiologicamente mais relevantes são: *C. albicans, Candida parapsilosis, Candida tropicalis, Candida glabrata, Candida krusei, C. guilliermondii* e *C. lusitaniae* (COLOMBO *et al*., 2013).

Candida albicans é a espécie mais frequententemente isolada em episódios de candidemia de todas as regiões do mundo (CAGGIANO *et al*., 2013; CLEVERLAND *et al*., 2015; KIM; SUDBERY, 2011; NUCCI *et al*., 2013; KREUSCH; KARSTAEDT, 2013; SANGUINETTI; POSTERARO; LASS-FLÖR, 2015; TAM *et al*., 2015). Essa levedura é polimórfica, capaz de formar hifa ou pseudo-hifa (MAYER; WILSON; HUBE, 2013; MOHANDAS; BALLAU, 2011). Essa transição anatômica é um fator de virulência que contribui para sua invasão e sobrevivência no hospedeiro (NAGLIK *et al*., 2011).

As espécies *C. tropicalis* e *C. parapsilosis* estão entre a segunda e terceira mais encontradas no Brasil, dentre os casos de candidemia. (BERGAMASCO *et al.*, 2015; MAGALHÃES *et al.*, 2015; NEVES-JÚNIOR *et al.*, 2015; OLIVEIRA *et al*., 2014; WILLE *et al*., 2013). *C. parapsilosis* chega a ser até mais frequente que *C. albicans* em alguns casos de candidemia e infecções nas mucosas (HOFFMANN-SANTOS *et al*., 2013). Essa espécie é muito comum em infecções na região genital, principalmente em pacientes do sexo feminino (BENTUBO *et al*., 2015). Também é responsável pela morte de neonatos, devido à passagem

transversal do fungo de mãe para filho no momento do parto (ARAÚJO PAULO MEDEIROS *et al*., 2014; CHERMONT *et al*., 2015).

Existe atualmente uma tendência ao aumento da frequência das espécies não-albicans. Oliveira *et al*., (2015) observou um aumento das espécies de *Candida* não-albicans em relação à *C. albicans*, entre crianças com candidemia com o passar dos anos, no sudeste do Brasil. Em 2007 essas espécies totalizavam 57,7% dos isolados clínicos e em 2010 foram 64%. Mesmo assim, *C. albicans* continua se destacando como a espécie mais comum.

C. *glabrata* e C. *krusei* aparecem com menor frequência neste país. Essas são as quartas e quintas espécies mais isoladas das infecções na corrente sanguínea (BERGAMASCO *et al.*, 2015; OLIVEIRA *et al*., 2014; WILLE *et al*., 2013). Entretanto, a frequencia com que se encontram as espécies de *Candida* varia muito de acordo com o clima e a região. Na América do Norte e Europa isolados de *C. glabrata* são mais comuns, ocupando o segundo lugar (DIEKEMA *et al*., 2012; PONGRÁCZ *et al*., 2015; SANGUINETTI; POSTERARO; LASS-FLÖR, 2015).

A espécie *C. guilliermondii* é também encontrada em menor freqüência e pertence ao grupo de espécies de *Candida* consideradas incomuns. Causa infecção sistêmica principalmente em pacientes oncológicos e com malignâncias hematológicas, como por exemplo, pacientes neutropênicos (GAMALETSOU *et al*., 2014; SAVINI *et al.*, 2011) Outra espécie incomum é *C. lusitaniae.* Essa levedura causa candidemia principalmente em pacientes com malignidades hematológicas, que receberam transplante de células e internados em UTI (ASNER *et al*., 2015; CANTÓN *et al.*, 2013). Jung *et al*. (2015) realizou um estudo no Texas que mostrou que a incidência de *C. lusitaniae* triplicou nos anos de 1998-2005 e 2006-2013 entre pacientes oncológicos.

Para o tratamento da candidíase o fármaco de primeira linha é o fluconazol. A anfotericina B também é um fármaco com atividade para *Candida* sp., no entanto devido à sua nefrotoxicidade, são os azóis que têm sido utilizados com mais freqüência (GIRI; KINDO, 2012; HOFFMANN-SANTOS *et al*., 2013). Por isso, autores relatam que o aumento da resistência aos antifúngicos está acontecendo principalmente ao fluconazol (DA COSTA *et al.*, 2014; HERKERT *et al*., 2015)

Algumas espécies apresentam uma maior resistência ou são intrinsecamente resistentes aos azóis como *C. glabrata* e *C. krusei* (BERGAMASCO *et al*., 2012; DA COSTA *et al*., 2014; NEUFRELD *et al*., 2015; WILLE *et al*., 2013). O uso desses antifúngicos pode

reduzir a carga microbiana de *C. albicans*, que é mais sensível, e levar a um aumento das espécies não-albicans devido à redução da competição (DIEKEMA *et al.*, 2012).

Em relação à *C. guilliermondii* e *C. lusitaniae*, pacientes acometidos por essas leveduras possuem certo grau de deficiência no sistema imunológico o que acaba resultando em uma candidemia persistente. A exposição prolongada aos agentes antifúngicos para combater essas doenças também dificulta o processo de cura, pois propicia o aparecimento de cepas com resistência adquirida (ASNER *et al*., 2015; GAMALETSOU *et al*., 2014; JUNG *et al.*, 2015). Já foram encontrados alguns isolados de *C. guilliermondii* menos susceptíveis ou resistentes aos antifúngicos poliênicos, triazóis e equinocandinas (COLOMBO *et al.*, 2013; JUNG *et al.*, 2015; SANGUINETTI; POSTERARO; LASS-FLÖR, 2015; SAVINI *et al*., 2011) e isolados de *C. lusitaniae* resistentes às equinocandinas, anfotericina B, flucitosina, e fluconazol (ASNER *et al*., 2015; ARENDRUP; PERLIN, 2014; PERLIN, 2011; ZHANG *et al.*, 2012).

2.1.2. *Cryptococcus* sp.

O gênero *Cryptococcus* é formado por leveduras encapsuladas pertencentes ao filo Basidiomycota e família Tremellaceae (HAGEN *et al.*, 2015). Esse gênero é composto por mais de 38 espécies, contudo, *C. neoformans e Cryptococcus gatti* são as espécies epidemiologicamente mais importantes (ESPINEL-INGROFF, 2012; HAGEN *et al*., 2015).

C. neoformans tem distribuição mundial, a principal fonte de contaminação desse fungo nos seres humanos é as fezes das aves, especialmente dos pombos (*Columba Lívia*) (LIMA; KLAFKE; XAVIER; 2015). *C. neoformans* tem a capacidade de colonizar a mucosa do papo dos pombos sem causar doença, sendo um parasita natural dessas aves. Esse é um fator que contribui para a manutenção e dissipação desse fungo nos centros urbanos (TEODORO *et al*., 2013). Por outro lado, *C. gattii* ocorre em regiões tropicais e subtropicais, e tem como habitat natural os materiais vegetais em decomposição e as árvores, como o eucalipto (*Eucalyptus camaldulensis*) (BARBOSA *et al*., 2013; LIMA; KLAFKE; XAVIER; 2015; SIDRIM; ROCHA, 2010).

Um fator de virulência de *Cryptococcus* spp. é a presença de uma cápsula constituída por polissacarídeos que aumenta sua patogenicidade, já que inibe a fagocitose e protege a levedura no interior dos fagócitos (XIE *et al*., 2012). Essa cápsula, também possui antígenos específicos que distinguem *C. neoformans* de *C. gattii* e os separam entre serotipos

diferentes. *C. neoformans* é dividido em serotipos A, D, e o serotipo híbrido AD e *C. gattii* em serotipos B e C. Ainda existem os serotipos híbridos AB e BD, que ocorrem entre as duas espécies (SPIES *et al*., 2014).

Essas leveduras são oportunistas e causam criptococose no pulmão e no cérebro. A infecção acontece pela inalação dos esporos que, dentro do organismo se estabelecem nos pulmões e se não forem eliminados pelo sistema imune do hospedeiro, podem se disseminar por via hematológica para os outros órgãos, incluindo o cérebro. O fungo pode penetrar no sistema nervoso central, potencialmente causando meningite (KIM *et al*., 2012). A criptococose ocorre em 1 milhão de pessoas por ano no Brasil e é responsável por aproximadamente 700.000 mortes (ALANIO; DESNOS-OLLIVER; DROMER, 2011). Dados epidemiológicos indicam que *C. neoformans* ocorre mais frequentemente em indivíduos imunocomprometidos, particularmente aqueles com síndrome da imune deficiência adquirida (SIDA). Em contraste, *C. gattii* é mais comum em indivíduos saudáveis (ALANIO; DESNOS-OLLIVER; DROMER, 2011).

Cogliati (2013) fez uma pesquisa bibliográfica a respeito do número de isolados de *Cryptococcus* sp. no mundo. O autor encontrou 68.811 isolados. As regiões mais comuns foram a Ásia e África (28,5%) seguida da América central e do Sul (15,5%), Europa (12,7%), América do Norte (11,5%) e Oceania (3,7%). A distribuição das duas espécies não é uniforme, uma vez que depende do espaço geográfico e do clima. Por exemplo, Barllet *et al*. (2012) realizou um estudo nas províncias da Columbia Britânica, situada no Canadá, entre 2004 a 2007 e observou que houveram 281 casos de *C. gattii*, contudo não houveram isolados de *C. neoformans.* Entretanto, Smith, *et al.* (2015) realizando uma pesquisa na Alemanha entre os anos de 1999-2010, observou apenas quatro casos de *C. gatti* e 141 casos de *C. neoformans*.

No Brasil, a maioria dos estudos epidemiológicos se concentra nas regiões sul, sudeste e centro-oeste. Tsujisaki *et al*. (2013) realizaram um estudo no Mato Grosso do Sul, com pacientes acometidos por criptococose no período de 1998 a 2012. *C. neoformans* foi a espécie mais frequênte (97,9%). A maioria dos pacientes era do sexo masculino (72,9%), foi diagnosticada com SIDA (81,3%), a faixa de idade foi entre 35-49 anos (31,3%) e a letalidade foi alta, 72,9%. Mezzari *et al*. (2013), realizando uma pesquisa em Porto Alegre no período de 2012 a 2013, obtiveram resultados parecidos. A maioria dos pacientes eram soro-positivo para SIDA (95%), a faixa etária foi de 30-39 anos (50%), e 55% dos pacientes eram do sexo

masculino. A idade dos pacientes acometidos por criptococose pode ter relação com a maior frequencia de SIDA nessa faixa etária.

Apesar dos estudos se concentrarem nas regiões anteriormente citadas, Martins *et al.* (2011) mostraram que essa doença também tem importância no nordeste do país, sendo endêmica nessa região, mais precisamente no Piauí. Dos isolados analisados, aproximadamente 60% eram *C. neoformans*, sendo que 87% destes foram obtidos de pacientes soro-positivos para SIDA. Os outros 40% eram *C. gatti* e 80% desses isolados foram encontrados em pessoas saudáveis. A letalidade foi também alta, aproximadamente 50% para os pacientes acometidos por ambas as espécies.

Alguns trabalhos focalizaram na fonte de infecção de *Cryptococcus*. Teodoro *et al.* (2013) avaliaram os isolados de *Cryptococcus* coletados de fezes de aves em São Paulo no período de 2009-2011. Das 87 amostras, 66,6% foram positivas para o gênero *Cryptococcus*. A espécie mais freqüente foi *C. neoformans* (17,2%) seguida de *C. gattii* (5,2%), *Cryptococcus ater* (3,5%), *Cryptococcus laurentii* (1,7%), e *Cryptococcus luteolus* (1,7%). O estudo de Leite *et al.* (2012), realizado no Mato Grosso, relatou um foco de infecção de *Cryptococcus* spp.advindo da poeira de livro de uma biblioteca pública. Das 84 amostras coletadas, 18 (21,4%) foram positivas para *Cryptococcus* spp. As espécies mais frequentemente isoladas foram *C. gattii*: 15 (36,6%); seguido por *C. terreus*: 12 (29,3%); *C. luteolus*: 4 (9,8%); *C. neoformans* e *C. uniguttulatus*: 3 cada um (7,3%); e *C. albidus* e *C. humicolus*: 2 cada um (4,6%).

Para o tratamento de meningite criptocócica é utilizado, como tratamento de primeira linha a anfotericina B, a flucitocina ou uma associação entre os dois de modo a conseguir uma diminuição da toxicidade da anfotericina B. Como tratamento de segunda linha, há alguns azóis que oferecem uma boa resposta a estas infeções, como exemplo, o fluconazol (mais comum), o itraconazol, o voriconazol e o posaconazol (GULLO *et al.*, 2013).

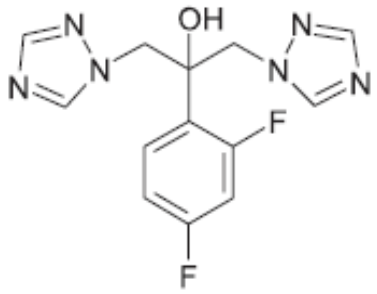
2.2. Atividade antifúngica de fluconazol

Os principais alvos da terapia antifúngica são a membrana plasmática, a parede, a divisão celular (COSTA; CURSI; NEVES, 2010) e a síntese de ácidos nucléicos (ARMSTRONG; TAYLOR, 2009). Os agentes antifúngicos disponíveis atualmente podem ser divididos em três principais classes: polienos e azólicos que são dirigidos seletivamente

para o alvo ergosterol na membrana celular dos fungos, e as equinocandinas que inibem a síntese de glicanos da parede celular (HARBISON *et al*., 2009).

O fluconazol é um triazólico, derivado azólico de segunda geração. Sua descoberta foi resultado de um programa de pesquisa dirigido ao desenvolvimento de um agente antifúngico de amplo espectro de ação, ativo pela via oral e intravenosa, para tratamento de infecções superficiais e sistêmicas (PEREIRA, 2007). É um medicamento fungistático, eficiente contra leveduras e fungos filamentosos (FUJISAWA, 2011; HARBISON *et al.*, 2009). Possui vantagem por ser dissolvido em água, por ser um medicamento de baixo custo e possuir poucos efeitos adversos (LI *et al*., 2014). Está disponível no mercado em cápsulas de 50, 100 e 150 mg e bolsa para infusão intravenosa de 2 mg/mL (COSTA; CURSI; NEVES, 2010).

Figura 1: Estrutura química do fluconazol



Fonte: Pereira, 2007.

Os compostos azólicos atuam inibindo a enzima 14α-esterol desmetilase, impedindo a biossíntese de ergosterol, componente da membrana fúngica (COSTA; CURSI; NEVES, 2010). A 14α-esterol desmetilase é uma enzima do citrocromo P450 que converte o lanosterol em ergosterol (ARMSTRONG; TAYLOR, 2009) e é codificada pelo gene ERG11. O antifúngico atua por meio do nitrogênio livre do anel azol. Esse interage com o ferro do grupo heme da 14α-esterol desmetilase, impedindo assim a reação enzimática (CURY, 2010). A redução da biossíntese do ergosterol leva à desestabilização da membrana fúngica, que em última análise acarreta na morte celular.

A respeito dos efeitos adversos do fluconazol, esse antifúngico tem sido associado a raros casos de toxicidade hepática grave (FUJISAWA, 2011). O fluconazol é seletivo para a enzima fúngica que utiliza o lanosterol como substrato, a 14α-esterol desmetilase, uma vez que nos mamíferos ela não é expressada. Entretanto, os agentes azólicos

são também inibidores de outras enzimas hepáticas do citocromo P450 o que explica essa toxicidade (ARMSTRONG; TAYLOR, 2009). Apesar disso, a hepatotoxicidade causada pelo fluconazol tem sido geralmente reversível com a descontinuação do tratamento.

A susceptibilidade *in vitro* entre as espécies de *Candida* em relação ao fluconazol não é uniforme. Sanguinetti, Posteraro e Lass-flör (2015) realizaram uma pesquisa bibliográfica envolvendo 41 países ao redor do mundo e descobriram que 90,2% dos isolados de *Candida* pesquisados foram susceptíveis ao fluconazol. Entretanto, 13 das 31 espécies analisadas tiveram menos que 75% das cepas sensíveis a esse medicamento e foram consideradas com susceptibilidade reduzida. As espécies mais comuns, *C. albicans*, *C. parapsilosis*e e *C. tropicalis* tendem a ter a CIM (concentração inibitória mínima) relativamente baixa, enquanto para *C. glabrata* tende a ser alta. *C. krusei* é intrinsecamente resistente ao fluconazol (EUCAST- Comitê europeu de teste de susceptibilidade antimicrobiana, 2013).

Entre as espécies de *Candida* incomuns, várias delas (*Candida ciferrii*, *C. guilliermondii*, *Candida inconspicua*, *Candida humicola*, *Candida lambica*, *Candida lipolytica*, *Candida norvegensis*, *Candida palmioleophila*, *Candida rugosa* e *Candida valida*) têm apresentado, até a data, menor suscetibilidade intrínseca aos azóis (PFALLER *et al*., 2010). Dentre essas, algumas cepas de *C. guillermondii* e *C. lusitaniae* têm se mostrado resistentes ao tratamento com fluconazol (JUNG *et al.*, 2015; ZHANG *et al.*, 2012).

O uso profilático ou o uso terapêutico prolongado (>30 dias) dessa droga tem selecionado as cepas menos sensíveis. Um estudo observou que amostras de *C. glabrata* recuperadas no sangue de pacientes que receberam tratamento prolongado com fluconazol apresentaram-se resistentes ao fármaco, e consequentemente, houve um aumento dos índices de re-infecção por esse fungo (SANGUINETTI; POSTERARO; LASS-FLÖRL, 2015).

2.3. Atividade antifúngica das estatinas

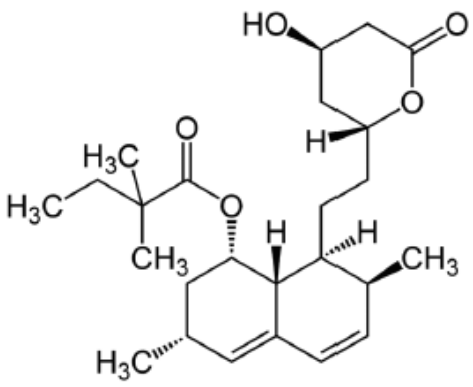
As estatinas são as mais prescritas dentre todas as drogas disponíveis no mercado e são utilizadas para reduzir níveis de colesterol sanguíneo (WIKHE; WESTERMEYER; MACREADIE, 2007). Dessa forma, acontece a diminuiçãoda deposição de gordura nos vasos, que consequentemente reduz o risco de arteriosclerose e de acidente cardiovascular (PESARO *et al.*, 2012). A sinvastatina é um tipo de estatina, é comercializada

na forma de comprimidos revestidos nas concentrações de 5, 10, 20, 40 e 80 mg (POLONINI *et al.*, 2011).

A sinvastatina é produzida a partir de um produto de fermentação do fungo filamentoso *Aspergillus terreus* (ENDO, 1979, 2004). De acordo com a ANVISA, (Agência Nacional de Vigilância Sanitária) essa droga é considerada um pró-fármaco porque está disponível em sua forma inativa e após ser ingerida é hidrolisada ao β-hidroxiácido. Esse é considerado o metabólito principal e age competindo pelo sítio ativo da enzima HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) redutase, inibindo-a. Essa inibição ocorre por uma competição seletiva, já que a HMG-CoA tem mais afinidade com a estatina do que com a HMG-CoA redutase (SEWRIGHT; CLARKSON; THOMPSON, 2007). Essa enzima catalisa a biossíntese do HMG-CoA em mevalonato, subsequentemente em farnesil difosfato (MACREADIE *et al.*, 2006). Odifosfato de farnesilo é o precursor para a produção de colesterol de seres humanos ou ergosterol em plantas e microrganismos eucarióticos (MACREADIE *et al.*, 2006). Dessa forma, o efeito inibitório das estatinas acontece em base na influência negativa sobre a fluidez da membrana (NYILASI *et al*., 2010).

Além de ser precursor do ergosterol, o farmesil difosfato é também precursor de CoQ (coenzima Q; ubiquinona) e heme A. Com a inibição desses, a sinvastatina afeta na captação de oxigênio e geração de energia, prejudicando a função mitocondrial. Nos fungos isso causa a morte celular e nos humanos miopatias, que podem levar à rabidomiólise (lesão no tecido muscular podendo levar a insuficiência renal aguda devido ao acúmulos dos resíduos células no sangue) (WIKHE; WESTERMEYER; MACREADIE, 2007).

Figura 2: Estrutura química da sinvastatina



Fonte: Poloniniet *al*., 2011.

Autores já reportaram a atividade das estatinas *in vitro* contra fungos patogênicos, como espécies de *Candida* (QIAO *et al*., 2007; WIKHE; WESTERMEYER; MACREADIE, 2007; LIU, *et al*., 2009; MENEZES *et al*., 2012), *C. neoformans*, zygomycetos (LORENZ; PARKS, 1990, CHAMILOS; LEWIS; KONTOYIANNIS, 2006) e *Aspergillus* spp. (QIAO *et al*., 2007). Além disso, estudos têm mostrado que as estatinas possuem atividade contra bactérias. Elas inibem a invasão celular de *Staphilococcus aureus* (HORN *et al.*, 2008) e de *S. pneumoniae*. Nesta última espécie foi demonstrado com testes *in vitro* e *in vivo* (BERGMAN *et al.*, 2011).

Autores também comprovam que as estatinas são eficientes contra protozoários. Grellier *et al*., 1994 comprovaram a inibição do desenvolvimento de *Plasmodium falciparum* e *Babesia divergens*. Outros pesquisadores mostraram a inibição do crescimento de *Trypanosoma cruzi* e várias espécies de *Leishmania* quando tratados com esse medicamento (SEALEY-CARDONA *et al*., 2007). Pradines *et al*. (2007) demonstraram que esse grupo farmacológico também interfere no crescimento de *P. falciparum* e Li *et al*. (2013) comprovaram a ação antimicrobiana de atorvastatina (uma estatina) contra *T. gondii.*

2.4. Uso de modelos alternativos na experimentação

A utilização de animais em experimentos científicos tem sido realizada desde o século V a.C. (RAYMUNDO; GOLDIN, 2002). Os modelos tradicionais utilizam animais vertebrados em suas metodologias, principalmente mamíferos (murinos, coelhos, cachorros, gatos e macacos) (FAGUNDES; TAHA, 2004). Esses animais são considerados sencientes, capazes de sentir emoções como angústia e sofrimento, pois, seu sistema nervoso central é capaz de ativar o sistema sensorial periférico (MILLS *et al*., 2010). Partindo dessa constatação, no início do século XIX começaram a surgir discussões éticas a respeito do uso de animais sencientes na pesquisa, quando foram criadas as primeiras sociedades protetoras dos animais (RAYMUNDO; GOLDIN, 2002).

Alguns fatos tomaram destaque na história: no início da década de 1930, Adolf Hitler depois de assumir o poder, publicou um decreto tornando a experimentação animal ilegal (American Medical Association, 1909). Em 1959, Russell e Burch publicaram o livro *The principles of humane experimental technique*, no qual afirmaram que a pesquisa consciente utilizando animais deveria respeitar três Rs: *replacement, reduction* e *refinement* (PETROIANU, 1996). Os três Rs objetivavam diminuir o número de animais utilizados na

pesquisa, diminuir a dor e o desconforto e buscar alternativas para substituição dos testes *in vivo* (CAZARIN; CORRÊA; ZAMBRONE, 2004).

Duas décadas mais tarde, em 1975, Peter Singer publicou o livro *Animal Liberation*, que relatava sobre as condições a que os animais eram submetidos pela indústria de cosméticos e no processo de produção de alimentos (SINGER, 1991). A partir desse momento, houve muitos protestos contra as indústrias de cosméticos, e as empresas REVLON, AVON e Bristol-Myers, passaram a colaborar para o desenvolvimento de modelos alternativos (RAYMUNDO; GOLDIN, 2002). Três anos depois desse acontecimento (1978) em uma reunião em Bruxelas foi estabelecido a Declaração Universal dos Direitos dos Animais a qual defendeu que qualquer tipo de experimentação animal que implique sofrimento físico ou psicológico seria incompatível com os direitos dos animais e também postulou que as técnicas de substituição deveriam de ser criadas e colocadas em uso.

A primeira regulamentação a respeito desse assunto no Brasil foi criada em 1934 (Decreto 24.645/34) e discorre sobre a proibição de maus tratos físicos aos animais durante a execução práticas escolares. A lei de Crimes Ambientais de 1998 é a que mais cita o assunto. Esta lei estabelece que seja crime praticar ato de abuso, maus-tratos, ferir ou mutilar animais silvestres, domésticos ou domesticados, nativos ou exóticos. Na mesma lei diz que "incorre nas mesmas penas quem realiza experiência dolorosa ou cruel em animal vivo, ainda que para fins didáticos ou científicos, quando existirem recursos alternativos".

Diante do conflito ético e pressão social, desde meados de 1800, pesquisas com modelos alternativos tem sido desenvolvidas, aumentando sua frequência a partir de 1960 (WILSON-SANDERS, 2011). Um dos modelos mais comuns são os invertebrados. Diferentemente dos animais vertebrados, não há evidência científica de que os hemicordados, alguns gastrópodes e moluscos, insetos não sociais, isópodas, equinodermos, anelídeos, platelmintos e nemátodos possam sentir dor e angústia. Já no caso dos tunicatos, aranhas e insetos sociais, alguns estudos indicam que esses animais são capazes de sentir dor e angústia, porém não conseguem entender para avaliar os perigos envolvidos nisso (Sociedade Européia de Segurança Alimentar, 2005).

Analisando o banco de dados eletrônicos do PubMed, Wilson-Sanders (2011) identificou um número crescente de invertebrados utilizados na pesquisa. De 1800 a 1900 apenas três artigos foram encontrados que utilizaram experimentalmente esse grupo de animais; já no período de 2008 a 2010 foram identificados 44 mil trabalhos. Os hospedeiros alternativos mais utilizados na pesquisa são a mosca da fruta *Drosophila melanogaster*, o

nematóide *Caenorhabditis elegans* e a mariposa parasita de colméia *Galleria mellonella* (WILSON-SANDERS, 2011). *D. melanogaster*, já tem seu genoma mapeado e sequenciado (GILBERT 2008). Essa espécie tem contribuído em estudos a respeito de comportamento, embriologia e doenças humanas (BECKINGHAM *et al*., 2005).

O sistema nervoso de *C. elegans* já foi mapeado (WHITE *et al.*, 1976) e possui algumas semelhanças em comparação aos humanos em nível genético e molecular. Devido a esse motivo é utilizado como modelo na área de genética, psicologia e biologia. Também já contribuiu em estudos sobre doenças como Alzheimer, Parkinson, diabetes e câncer (NASS; MERCHANT, RYAN, 2008). As larvas de *G. mellonella* têm encontrado aplicações generalizadas na pesquisa de doenças infecciosas (DESALERMOS; FUCHS; MYLONAKIS, 2012) e no estudo sobre a demência, acidente vascular cerebral, câncer e diabetes (BRENNAN *et al.*, 2002).

Quando se trata de memória, aprendizagem e comportamento, muitos outros invertebrados já contribuíram para o nosso conhecimento nessa área. Essa gama de animais abrange desde lesmas, polvos, moluscos, náutilos, crustáceos e abelhas. Essas últimas, por exemplo, podem ser usados até em pesquisas comportamentais por se comunicarem através da dança. (WILSON-SANDERS, 2011). Os animais invertebrados também contribuem para a investigação sensorial como a visão, olfato, toque e paladar. Em relação ao olfato, apesar de utilizarem antenas como órgão olfativo, há muitas semelhanças estruturais, funcionais e fisiológicas entre elas e o nosso nariz (KAY; STOPTER, 2006).

Até mesmo embriões de animais vertebrados podem ser considerados modelos alternativos, apesar de necessitarem de aprovação pelo conselho de bioética. Ovos embrionados são muito utilizados na pesquisa de vacinas antivirais e nos diagnósticos de viroses (GUY, 2015). O embrião do peixe zebrafish (*Danio rerio*) é o único modelo utilizado para estudos onde se pode visualizar a evolução da infecção e entender sua interação com o sistema imune do hospedeiro. Uma vez que o embrião é relativamente translúcido, a visualização acontece por meio de um marcador, sem necessitar de métodos invasivos (BROTHERS; NEWMAN; WHEELER, 2011).

Um modelo alternativo que já é utilizado com mais frequência é a cultura de célula, importante no estudo de vírus, por serem intracelulares obrigatórios, e de protozoários por também se desenvolverem dentro das células (LI *et al*., 2015; RIBEIRO-GOMES *et al*., 2015). Órgãos viáveis de animais que foram sacrificados para a alimentação humana ultimamente não estão sendo mais descartados. Córnea bovina que seria inutilizada está

servindo para teste de opacidade de córnea bovina e olhos de coelho e galinha estão sendo utilizados em testes de edema e opacidade de córnea bem como a retenção de fluorescência (MORALES, 2008). Outros modelos que estão surgindo são os micro-organismos que são aceitos em estudos envolvendo o metabolismo, genética e bioquímica; e simulações computacionais que formam redes neurais artificiais auxiliando no estudo sobre o sistema nervoso (TEJKOWSKI, 2013).

2.4.1. *Galleria mellonella*

G. mellonella é uma mariposa (ordem Lepidóptera) de tamanho médio, cujas larvas vivem em colméias, alimentando-se das ceras dos favos. Ela pertence à família Pyralidae, e subfamília Galleriinae (GALLO *et al*., 2002). O uso dessas larvas como modelo alternativo tem encontrado aplicações generalizadas tanto na pesquisa de doenças infecciosas causadas por bactérias e fungos (DESALERMOS; FUCHS; MYLONAKIS, 2012) quanto não-infecciosas: a demência, acidente vascular cerebral, câncer, diabetes (BRENNAN *et al*., 2002). Por ter significante vantagens ética, logística e econômica sobre os modelos de mamíferos (LI *et al.*, 2013) e ser passível de testes de elevado rendimento, em larga escala e com baixo custo (LIONAKIS, 2011), ela fornece uma avaliação rápida da eficácia e da toxicidade de agentes *in vivo* (LI *et al*., 2013).

Umas das vantagens é que o inseto pode ser mantido a uma temperatura entre 25 °C a 37 °C, que o torna adequado para estudar agentes patogênicos na temperatura do corpo humano (LI *et al*., 2013). É uma vantagem em relação a *D. melanogaster* e *C. elegans*, que não toleram temperaturas tão altas, sobrevivendo apenas à temperatura ambiente (DESALERMOS; FUCHS; MYLONAKIS, 2012). Nesses modelos, os fungos não são estudados nas condições em que são patogênicos para os humanos e essas variações de temperatura podem afetar a expressão dos genes (FUCHS *et al.*, 2010).

O uso de *G. mellonella* em testes de infecção com bactérias, fungos leveduriformes e filamentosos é encontrado na literatura. A larva da mariposa da cera foi utilizada para avaliar a patogenicidade de *Pseudomonas aeruginosa* (MIYATA *et al.*, 2003) e em testes de compostos antibacterianos contra a mesma (ANDREJKO; MIZERSKA-DUDKA; JAKUBOWICZ, 2009). Hoffmann, Hultmark e Boman em 1981 avaliaram a resposta imune de *G. mellonella* contra *Enterobacter cloacae e Escherichia coli*.

Em relação aos fungos, larvas dessa mariposa já serviram como modelo de infecção na avaliação da virulência de *Candida* spp (COTTER; DOYLE; KAVANAGH, 2000), *Cryptococcus neoformans* (MYLONAKIS, 2005), *Aspergillus fumigatus* (RENWICK *et al*., 2006; REEVES *et al*., 2004), *Aspergillus flavus* (SCULLY; BIDOCHKA, 2005) e *Fusarium oxysporum* (DESALERMOS; FUCHS; MYLONAKIS, 2012). Pesquisas mostraram que há correlação entre a virulência de *P. aeruginosa* (JANDER; RAHME; AUSUBEL, 2000) e *C. albicans* (BRENNAN *et al*., 2002) em camundongos e em larvas de *G. mellonella*.

2.4.2. *Tenebrio molitor*

Tenebrio molitor (Coleoptera) é um besouro que tem importância na agricultura por ser considerado praga de grãos como o trigo, por exemplo (SCHROECKENSTEIN; MEIERDAVIS; BUSH, 1990). Em estágio larval é utilizado na alimentação de animais de estimação, especialmente as aves (BARKER *et al*., 1998). Contudo, em países principalmente asiáticos é utilizado também na alimentação humana (VAN BROEKHOVEN *et al*., 2015). Esse inseto possui distribuição mundial (RAMOS-ELORDUY *et al*., 2002) e assim como *G. mellonella*, tolera temperatura de 37 °C (LI *et al*., 2013). Também possui vantagem logística em relação a modelos utilizando murinos, além de não precisar de aprovação pela comissão de bioética (LI *et al*., 2013; LIONAKIS, 2011; OLIVEIRA; GOLDIN, 2014).

A criação de *T. molitor* em condições laboratoriais é mais fácil em comparação com *G. mellonella*. Além disso, há vários fornecedores comerciais de desse inseto no Brasil, enquanto que *G. mellonella* não é comercializada neste país, obrigando os investigadores a manterem o seu próprio fornecimento de larvas da mariposa para os experimentos. Isto eleva os custos dos experimentos e requer uma pessoa responsável pela sua manutenção. Outra vantagem refere-se à forma de inoculação, que é realizada por injeção tanto no modelo com *T. molitor* quanto com *G. mellonella* (OPPERT *et al*., 2012). No modelo com o verme *C. elegans*, no entanto, o agente patogênico é adicionado ao poço em que o nemátode é inoculado durante o experimento, assim não há exatidão de quantos microrganismos conseguirão infectar o animal (MERKX-JACQUES *et al*., 2013).

T. molitor teve seu transcriptoma recentemente publicado (OPPERT *et al*., 2012), oferecendo oportunidades para avanços da biologia molecular neste domínio. Esse

besouro já foi utilizado como hospedeiro alternativo em um estudo que comprovou que os processos inflamatórios e imunológicos aceleram o envelhecimento (PURSALL; ROFF, 2011). Ele também já foi utilizado no estudo de infecções com *Staphylococcus aureus* e *Listeria monocytogenes* (DORLING; MORAES; ROLFF *et al*., 2015; TINDWA *et al.*, 2013). No entanto, até a publicação do nosso artigo, não havia relatos sobre o uso das larvas do besouro como modelo para fungos patogênicos humanos.

2.4.3. Sistema imunológico de *G. mellonella* e *T. molitor*

Estudos a respeito do sistema imunológico de *T. molitor* são encontrados na literatura. Assim como os outros insetos, o besouro não possui resposta imune adaptativa, contudo, sua resposta imune inata pode ser de longa duração, mesmo quando não há infecção, proporcionando um efeito profilático, sendo funcionalmente parecida com a resposta imune adaptativa humana (MORET; SIVA-JOTHY, 2003). Além disso, os insetos possuem uma resposta imune sistêmica, que os auxilia no combate a infecções recorrentes.

Na resposta imune sistêmica de *T. molitor*, esse inseto produz diversos peptídeos antimicrobianos que são específicos para cada tipo de microrganismo. Existe uma classe desses peptídeos que são as proteínas tenecinas, que são produzidas na defesa contra fungos (JOHNSTON; MAKAROVA; ROLFF, 2014). Diferentes tipos delassão encontradas contra *C. albicans* (Kim, 2001; Lee *et al.*, 1999;) e contra bactérias gram-negativas ou gram-positivas (ROH *et al*, 2009), tais como *Escherichia coli* (CHAE *et al*., 2012) ou *Staphylococcus aureus* e *Listeria monocytogenes* (DORLING; MORAES; ROLFF, 2015; TINDWA *et al*., 2013).

A respeito de *G. mellonella*, seu genoma ainda não foi completamente sequenciado (LIONAKIS, 2011). Entretanto, há muito tempo já foram caracterizadas das sequencias dos genes que transcrevem o aparato imunológico da mariposa, o que levou ao conhecimento de microarranjos de genes e abriu o caminho na elucidação da sua resposta imune inata e de seus mecanismos antifúngicos (DUNPHY; THURSTON, 1990). Através desses estudos também foipossível afirmar que a resposta imune de *G. mellonella* e dos outros insetos em geral é semelhante ao de mamíferos e consiste de barreiras estruturais e passivasbem como de respostas celulares e sistêmicas, assim como foi publicado a respeito de *T. molitor* (DUNPHY; THURSTON, 1990).

As respostas celulares são realizadas por hemócitos encontrados na hemolinfa (DUNPHY; THURSTON, 1990). Já se sabe que a resposta imune dos insetos aos microrganismos envolve uma mudança na população de hemócitos circulantes e síntese de novas proteínas (MORTON; DUNPHY; CHADWICK, 1987). Há seis classes de hemócitos que são os prohemócitos, coagulócitos, esferulócitos, oenocitóides, plasmatócitos e granulócitos (LIONAKIS, 2011) e estão envolvidos na fagocitose, encapsulamento e formação de nódulos (MATHA; MRACEK, 1984). Esses são elementos importantes da defesa celular do inseto contra bactérias e fungos unicelulares (WALTERS; RATCLIFFE, 1983). Os parasitas maiores são encapsulados, e quando há um grande número de microrganismos invasores, ocorre a formação de nódulos (DUNN, 1986).

Outros fatores sistêmicos envolvidos na imunidade dos insetos frente à uma infecção, incluem a produção de lisozima, as lectinas e a cascata profenoloxidase (DUNPHY; THURSTON, 1990). Essa última induz a melanização do animal e isso acontece pela ativação de profenoloxidase em sua forma ativa fenoloxidase, uma enzima-chave que leva à formação de melanina em locais de feridas e ao redor de microrganismos que penetraram na hemolinfa.

Avanços nos estudos sobre a imunidade de *Drosophila* e outros insetos descrevem uma possível ligação entre a fenoloxidase e o sistema de coagulação, embora os mecanismos moleculares exatos que controlam esta interação parecem serem complexos e ainda não estão bem definidos (ELEFTHERIANOS; REVENIS, 2011). A coagulação é fundamental para limitar a perda de hemolinfa e iniciar a cicatrização de feridas após lesão e atua rapidamente para formar uma barreira contra a infecção imobilizando e promovendo a morte dos microrganismos (BIDLA *et al*., 2005).

Diante da importância clínica do gênero *Candida* e *Cryptococcus*, devido a todos os aspectos epidemiológicos associados às infecções por essas leveduras (como aumento da incidência, alta letalidade e internação prolongada) se fazem necessário a procura de diferentes classes de antifúngicos e modelos alternativos de infecção.

3. OBJETIVOS

3.1. Objetivo geral

Estudar as infecções fúngicas e avaliar a ação de antifúngicos utilizando modelos invertebrados.

3.2. Objetivos específicos

- Avaliar a atividade antifúngica *in vitro* de sinvastatina contra espécies incomuns de *Candida*;
- Verificar a taxa de sobrevivência de *G. mellonella* infectada com *C. albicans* e tratada com sinvastatina e fluconazol;
- Desenvolver um novo modelo invertebrado para o estudo de infeções fúngicas, utilizando larvas do besouro *T. molitor*.

4. REFERÊNCIAS

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5. RESULTADOS E DISCUSSÕES

Os resultados e as discussões, assim como as metodologias referentes a cada trabalho, da presente dissertação foram apresentados na forma de artigos (Artigo I, Artigo II e Artigo III) cada qual seguindo as normas de formatação da revista para qual foram ou serão enviados.

5.1. ARTIGO 1: Será enviado à revista Mycoses:

Simvastatin shows antifungal activity *in vitro* against uncommon *Candida* species

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Simvastatin inhibits uncommon *Candida* species

Key words: *Candida lusitaniae*, *Candida guilliermondii*, synergism, antifungal, addiction, simvastatin, fluconazole, uncommon, interaction.

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ABSTRACT

Candida guilliermondii and *Candida lusitaniae* are uncommun species of the genus *Candida* which are important to be associated with cases of breakthrough candidemia. Currently there are no many antifungal classes, so it is necessary search for new drugs. Simvastatin is a drug used in therapy for lowering blood cholesterol that proved to have antifungal activity in previous studies. So this work aimed to investigate the antifungal efficacy of simvastatin and its interaction with fluconazole *in vitro* against *Candida guilliermondii* and *Candida lusitaniae*. Antibiogram and broth microdiluition tests were made. In disk diffusion test *C. lusitaniae* formed halo with 25 μg of simvastatin and *C. guilliermondii* with 350 μg, and there was a positive interaction between the drugs. It also was required a higher concentration of simvastatin to inhibiting the growth of *C. guilliermondii* in broth microdilution test. *C. guilliermondii* showed the minimum inhibitory concentration of 312.5 μg/mL, ten times higher than *C. lusitaniae*. However, the interaction of the drugs for *C. guilliermondii* was synergistic while for *C. lusitaniae* was additive, showing that simvastatin can aid fluconazole in the treatment of infection due to the first fungal. So, simvastatin proved to inhibiting the growth of *C. guilliermondii* and *C. lusitaniae*.

Key words: *Candida lusitaniae, Candida guilliermondii*, synergism, antifungal.

1. INTRODUCTION

Candida guilliermondii and *Candida lusitaniae* are uncommon species of the genus *Candida* and occur mainly in patients with hematologic malignancies such as hypogammaglobulinaemia, neutropenia, leukopenia and presence of the intravenous catheter (1-4). Because these patients have certain level of deficiency in the defense mechanisms against pathogens, this aggravating factor contribute to the breakthrough candidemia and the extended exposure to antifungal agents difficult the cure process, therefore they provide the occurrence of strains with resistance acquired (1, 3, 5). Authors report the existence of some isolates of *C. guilliermondii* and *C. lusitaniae* resistant to polyene antifungals, triazoles and echinocandins (1, 4-9).

There are not many antifungal classes available (10) so lately researchers and health professionals have been concerned to search for drugs with different targets for action. A drug that has been studied is simvastatin, produced by the mold *Aspergillus terreus* (11, 12) that acts by reducing low density lipoprotein cholesterol levels in the blood (13). Statins work by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), which operates in mevalonate biosynthesis, precursor of steroids (14). Thus it decreased levels of ergosterol of fungal membrane, which ultimately affects the spread of fungi (26).

Some studies showed its action against filamentous fungi and yeasts (10, 15-20). Simvastatin proved able to inhibit the growth of several species of the genus *Candida*, however, *C. guilliermondii* and *C. lusitaniae* have not been studied. Most of these authors mentioned above tested statins in association with other antifungals and it has been proven synergy between simvastatin and fluconazole (10, 15, 17, 20). The azoles are compounds which act at the ergosterol synthesis through inhibition by another route of action. An advantage of the synergistic interaction between these two classes of medications is the low toxicity of statins to humans, compared with azoles (21). Therefore, this study aimed to test simvastatin and its activity interaction with fluconazole against *C. guilliermondii* and *C. lusitaniae*.

2. MATERIANS AND METHODS

2.1. Microorganisms

It was selected for this study a standard strain, *C. guilliermondii* ATCC 6260 and a clinical strain *C. lusitaniae* that was gently provided by the do Minho University, Braga, Portugal. *Candida parapsilosis* (sensu stricto) ATCC 22019 was used as control. The strains were maintained as stock cultures from the Mycology and Alternative Methods to Animal Use Laboratory, University of Londrina – Brazil, on Yeast Peptone Dextrose - YPD (3% yeast extract, 1% peptone, 1% glucose) at -80 °C in 40% glycerol.

2.2. Disk Diffusion test

The disk diffusion test was performed according to the standards of M44-A2 document of the Clinical and Laboratory Standards Institute (CLSI) (22). The strains were grown on YPD broth for 16-18 h at 30 °C shaking at 180 revolutions per minute (rmp). Then, a suspension of 10^6 colony forming units/mL (cfu/mL) was prepared by diluting in Phosphate Buffered Saline - PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). 100 µL of this suspension was placed on plaques containing YPD agar (3% yeast extract, 1% peptone, 1% glucose and 2% agar). After that, disks containing 25 µg of Fluconazole (Sigma-Aldrich) or 12.5, 25, 50 and 350 µg of simvastatin (SM handled by Pharmaceutical LTD) were placed on the plaques.

To show the interaction of the drugs on disk diffusion test, two disks with each drug were placed on the plaque and the distance between these disks was calculated considering the size of the halos previously measured, according to following formula:

$$\text{Distance (mm)} = \left[\left(\frac{\text{Halo diameter (sim)}}{2}\right) + \left(\frac{\text{Halo diameter (flu)}}{2}\right) + \text{ disks diameter}\right] - 10$$

Disks containing 10, 100 and 350 µg of simvastatin and 5, 10 and 50 µg of fluconazole were used. The drugs were prepared as follows: fluconazole was diluted in 0.5 mg/mL dimethylsulfoxide (DMSO) according to the manufacturer's instructions. Simvastatin

was hydrolyzed with 0.25 M NaOH at 35° C for 1 hour for activation, and then its pH was adjusted with 0.25 M HCl, adapted from Lorenz & Parks (23).

After 24 h of incubation at 35° C the reading was done by measuring the inhibition zone. According to M44-A2 document, strains of *Candida* spp. having halos higher or equal to 19 mm were considered susceptible (S), strains presenting halos between 15 and 18 mm were considered sensitive dose-dependent (SDD), and smaller halos or equal to 14 mm were considered resistant (R). *C. parapsilosis* ATCC 22019 was considered sensitive presenting halo 22-33 mm.

2.3. Testing broth microdilution

Antimicrobial susceptibility tests were performed according to the document M27-S4 (24) CLSI with adaptations. The strains were cultured in Sabouraud Dextrose broth (4% dextrose, 10% peptone, pH 5.6) for 16-18 h at 30° C shaking at 180 rpm (rotations per minute). It was used a 96 well plaques with background "u" and RPMI-1640 medium (Gibco) [added phenol red with glutamine, without bicarbonate, buffered with MOPS (3-(*N*-morpholino) propanesulfonic acid, with final concentration of 0.165 mol/L and pH 7.0].

Serial dilutions of fluconazole were made with concentrations ranging from 0.24-125 μg/mL and simvastatin with concentrations ranging from 0.24-1,250 μg/mL. The inoculums were prepared with 10^4 cfu/mL, and 100 μL of the suspensions were added to each well. The plaques were incubated at 35 °C and two visual readings were taken at 24 and 48 h to find the MIC 50. The interpretation of the results to fluconazole to *C. parapsilosis* was performed according to the recently revised CLSI clinical breakpoint which consider S $\leq$ 2 μg/mL, SDD = 4 μg/mL and R $\geq$ 8 μg/mL. The rare strains do not have a breakpoint established yet, so, the breakpoint determined was based to *C. parapsilosis* ATCC 22019.

Checkerboard (CB) technique (25) was done to analyze the interaction of the drugs. In this test, serial dilutions were made with simvastatin in the vertical position and subsequently, serial dilutions with fluconazole were made in horizontal. The concentrations of fluconazole were raging from 0.0075-31.25 μg/mL and simvastatin 0.12-62.5 μg/mL. 100 μL of a suspension containing 10^4 cfu/mL of fungi were inoculated and the readings were made according to the above test. The results of CB test were reached calculating fractional inhibitory concentration (FIC) (26), in which the sum of MIC of the drugs in combination is divided by the MIC of the drugs alone corresponding to the FIC, and the interpretation was

made as follows: ≤ 0.5, synergy; > 0.5 to 1.0, addition; > 1.0 to < 4.0, indifference; and ≥ 4.0, antagonism.

2.4. Statistical analysis

The standard deviation of repetitions of the two tests was measured. To compare the size of the halos of treatment with 25 μg of fluconazole and simvastatin it was used the Student t test. This statistical test was also used to compare the concentration of the drugs alone and in combination, p values <0.05 were considered significant.

3. RESULTS

In disk diffusion test, the strains were sensitive to fluconazole. The inhibition zones were formed when it was placed a minimum of 25 μg of simvastatin for *C. parapsilosis* ATCC 22019 and *C. lusitaniae* (Fig. 1). There was statistical difference ($p<0.05$) compared to the size of the halos in this concentration between the two drugs. It was necessary 350 μg of simvastatin to inhibit *C. guilliermondii* ATCC 6260 (Fig. 1). When two disks containing the drugs were placed near each other, it was found a positive interaction between the agents that may be additive or synergistic, as halos were fused (Fig. 2).

In microdilution broth test, all strains were sensitive to fluconazole, however, *C. parapsilosis* ATCC 22019 was sensitive dose-dependent at 48 h. Simvastatin was able to inhibit the growth of the three isolates, however, *C. guilliermondii* ATCC 6260 showed a MIC about 10 times greater than the other strain. The CB test reinforces the results of the interaction in the disk diffusion test. There was synergism for *C. gulliermondii* ATCC 6260. There was an additive effect at 24 h for *C. parapsilosis* ATCC 22019 and synergism in 48 h. For *C. lusitaniae*, there was added effect of the drugs (Table 1). Comparing the concentration of the drugs alone and in co-administration, there was a statistically significant reduction ($p <0.05$) for the tested strains.

4. DISCUSSION

The antifungal activity of simvastatin has been reported by some authors against filamentous fungi as zygomycetes and *Aspergillus* sp. (10, 16, 18, 19). Also, there

already been studies of its action against yeasts, including *Candida* species that have a higher incidence, such as *C. albicans* (10, 17, 19, 20). Authors have also studied the efficiency of this medication against rare strain of *Candida* (*Candida utilis*) and against yeast that is not considered pathogenic (*Saccharomyces cerevisiae*), however, in immunocompromised individuals it may manifest (15, 23).

In this work it was showed that simvastatin was able to inhibit the growth of two uncommons species of *Candida.* In a preliminary test using the disk diffusion method, it was necessary 25 μg of simvastatin to inhibit the growth of *C. lusitaniae* and a much greater quantity (350 μg) to control the growth of *C. guilliermondii* ATCC 6260. This finding was confirmed by microdilution broth test in which the MIC of *C. guilliermondii* ATCC 6260 was 312.5 μg/mL, ten times greater than the MIC found for the other strain (31.25 μg/mL). Other authors also have found variations in MIC between different *Candida* species (10, 17, 20). These data underscore the importance of specific study for each specie.

The disk diffusion test showed a positive interaction between the drugs, which could be synergy or addition. This was confirmed through the value of the FIC and the statistically significant reduction of the concentration of the drugs. Although it has been required a high concentration of simvastatin to inhibit *C. guilliermondii* ATCC 6260 growth, the interaction of the drugs was synergistic for this strain, different from what happened with the other, in which the effect was additive. It shows that even if the MIC for *C. guilliermondii* have been much higher than for *C. lusitaniae*, simvastatin can aid fluconazole in the treatment of infection due to the first fungal.

The co-administration of two antifungal compounds can enhance the efficacy of treatment extend the spectrum of action, reducing the resistance and the adverse effect due to reduced concentrations of the chemotherapeutic agents (26). An explanation for this synergistic effect is that simvastatin slightly reduces the ergosterol level produced by the fungus and ultimately inducing the increase in membrane permeability in order to elevate the absorption of exogenous ergosterol, thereby facilitating the entry of fluconazole (15). An advantage of the synergistic interaction between fluconazole and simvastatin is the low toxicity to humans statins compared with drugs of the class of azoles (15).

In conclusion, it was necessary a concentration ten times higher of simvastatin to inhibit the growth of *C. guilliermondii* ATCC 6260 in comparison to *C. lusitaniae*. However, this drug shows synergistic effect when administered with fluconazole and tested with the first fungus and it shows additive effect to the second specie. Therefore, this study

indicates that simvastatin has a good chance of becoming a new antifungal and the association with fluconazole, in low concentrations, may be a considerable alternative to reduce the fungal resistance and adverse effects of drugs.

5. ACKNOWLEDGEMENTS

The authors thank the project's funding agency, National Council for Scientific and Technological Development (CNPq, Brazil), and the Higher Education Personnel Improvement Coordination (CAPES, Brazil) for supplying the master's scholarships.

6. CONFLICT OF INTEREST

There was no conflict of interest.

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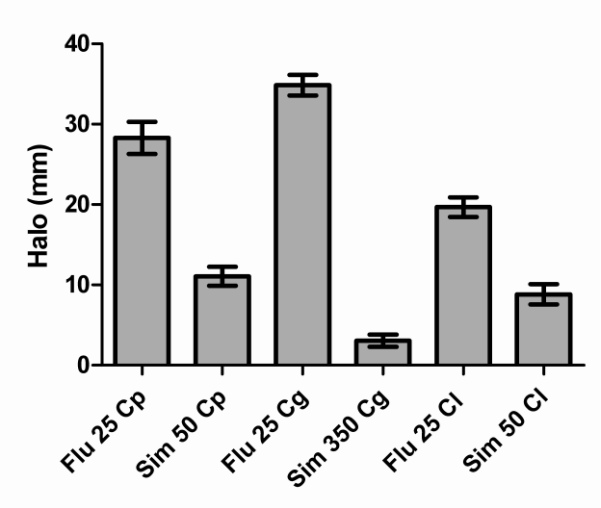
8. CAPTIONS

Figure 1 - Disk diffusion test results showing the size of the halos in relation to the concentrations of drugs. Cp - *C. parapsilosis*, Cl - *C. lusitaniae*, Cg - *C. guilliermondii*, Flu - fluconazole, Sim - simvastatin, mass given in μg.

Figure 2 - Disk diffusion test: plating fluconazole in the left disk and simvastatin in the right disk, showing a positive interaction of the drugs by the fusion of the halos (black arrows). *C. lusitaniae* with treatment with 5 μg of fluconazole and 10 μg of simvastatin, 20 mm distance between the disks. B: *C. guillermondii* ATCC 6260 treated with 10 μg of fluconazole and 350 μg of simvastatin, 12 mm distant between the disks. C: *C. parapsilosis* ATCC 22019 treated with 50 μg of fluconazole and 100 μg of simvastatin, 18 mm distancing disks.

Table 1 - Minimum inhibitory concentration of simvastatin and fluconazole, alone and in combination with reading performed at 24 and 48 h and their interpretations. MIC values were expressed in μg/mL.

Figure 1:



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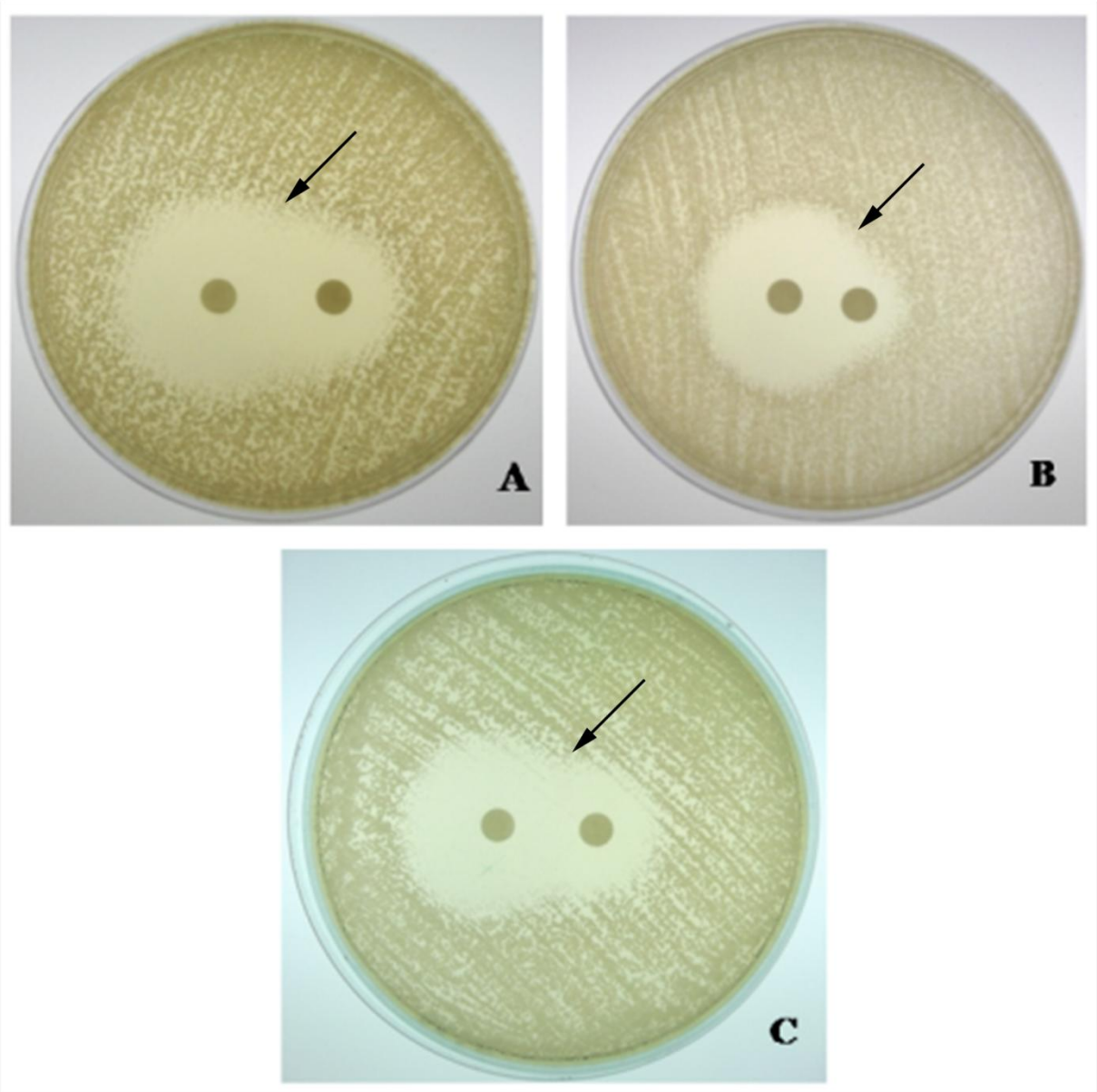

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Table 1 - Minimum inhibitory concentration of simvastatin and fluconazole, alone and in combination with reading performed at 24 and 48 h and their interpretations. MIC values were expressed in μg/mL.

Strain	MIC Sim (alone) 24h/48h	MIC Sim (combination) 24h/48h	MIC Flu (alone) 24h/48h	MIC Flu (combination) 24h/48h	FIC	Int
C. parapsilosis ATCC 22019	31.25/62.25	15.6/15.6	1.95/3.9	0.48/0.97	0.75/0.5	Add/Syn
C. gluilliermondii ATCC 6260	312.5/625.0	31.25/31.25	0.97/1.95	0.24/0.48	0.35/0.29	Syn/Syn
C. lusitaniae	31.25/31.25	3.9/3.9	0.24/0.97	0.012/0.06	0.75/1.0	Add/Add

FIC - fractional inhibitory concentration, MIC - minimum Inhibitory concentration, Syn - sinergia, Add - addition, Sim - simvastatin, Flu - fluconazole, Int - interaction, ATCC - American Type Culture Collection.

5.2. ARTIGO 2: Será enviado à revista Mycoses:

Simvastatin did not shows antifungal activity *in vivo* against *Candida albicans* in the *Galleria mellonella* infection model

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Simvastatin not shows antifungal action *in vivo*

Key words: *Galleria mellonella*, synergism, antifungal, simvastatin, fluconazole, *Candida albicans*, *in vivo*, invertebrate host, no correlation.

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ABSTRACT

The frequency of fungal infections is growing actually and the most common specie is *Candida albicans*. There is no many antifungal classes, so it is important the search for new substances with antifungal activity. A potential drug is simvastatin, medicament prescribed in the treatment of hypercholesterolemia that acts by inhibiting the biosynthesis of sterols, including the fungus membrane ergosterol. We evaluate its activity in combination with fluconazole *in vitro* and *in vivo* against *C. albicans* using as host *Galleria mellonella* larvae. Disk diffusion test, broth microdiluition test and survival assay were made. Simvastatin inhibited the fungal growth *in vitro* and showed a positive interaction with fluconazole in the first test. It was confirmed in broth microdilution test, which showed synergism between the drugs. In the survival assay, 80% of the larvae survived in 72 h when they received treatment with 50 mg/kg of fluconazole, however, simvastatin showed no protective response. When administered the drugs in combination, it resulted in the death of approximately 90% of the larvae at 150 h. This work was the first to test the antifungal activity of simvastatin *in vivo* and demonstrated that there is no correlation between the *in vitro* tests.

Key words: *Galleria mellonella*, synergism, antifungal.

1. INTRODUCTION

Candida genus is the most commonly isolated from fungal infections (1-3). Furthermore, the incidence of infections caused by *Candida* species has increased in recent years (4). The most common and important specie is *Candida albicans* (1, 4, 5-7). It is opportunistic yeast that develops superficial infections in healthy patients that may be cutaneous, subcutaneous or mucosal (8, 9). In immune compromised people due to cancer, AIDS (acquired immunodeficiency syndrome), diabetes or others risk factors, this pathogen can also cause disseminated infection (10).

The treatment of infections caused by opportunistic fungi is often complicated and the number of available antifungal agents is limited (11). The increased incidence of fungal infections leads to increased use of antifungal agents resulting in a higher rate acquired resistance (12). Faced with this fact, the search for new compounds with antifungal activity becomes important. A potential drug is simvastatin, which belongs to the class of the statins, the most prescribed in the treatment of hypercholesterolemia. It inhibits the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes the biosynthesis of mevalonate, precursor of cholesterol (13). However, this drug has also been used in *in vitro* tests for inhibit clinical fungi (14-16), whereas the mevalonate is also a precursor of the fungus membrane ergosterol.

In spite of that, until this moment there is no experimental test *in vivo* showing the antifungal activity of simvastatin. In this work it was chosen as the model host larvae of *Galleria mellonella* (Lepidoptera: Pyralidae), a wax moth, an alternative model to the use of vertebrate animals. Insect hosts have advantages over these traditional models, since they do not require the approbation of the bioethic committee. The creation and production is easier, less expensive and can be done on a large scale (17). *G. mellonella* host is standardized, Brennan *et al*. (18) found that there is correspondence between virulence of *C. albicans* in mouse host and in *G. mellonella* host. According to Li *et al.* (19) this wax moth provides *in vivo* a rapid assessment of the efficacy and toxicity of antimicrobials.

An alternative to reduce the fungal resistance is the combination therapy with two or more antifungals. It can improve the effectiveness of treatment broadening the activity spectrum, in addition, the resistance can be avoided and reduced toxicity using lower concentrations of the chemotherapeutic agents (20). In our study we evaluated the antifungal combination of simvastatin with fluconazole *in vitro* and *in vivo* against *C. albicans*, using as host larvae of *G. mellonella*.

2. MATERIALS AND METHODS

2.1. Strains and culture conditions

The strain that was used in the experiment was *C. albicans* ATCC 90028. It was maintained as stock culture from the Mycology and Alternative Methods to Animal Use Laboratory, State University of Londrina – Brazil, on Yeast Peptone Dextrose - YPD (3% yeast extract, 1% peptone, 1% glucose) at -80 °C in 40% glycerol.

The inoculums preparation was made in Sabouraud-Dextrose medium (4% dextrose, 10% peptone, pH 5.6) for *in vitro* tests and YPD for survival assay test. The fungus was incubated for 16-18 h at 30° C shaking at 180 rotations per minute (rpm).

2.2. Susceptibility testing by disk diffusion

The method of antibiogram test by disk diffusion was conducted according to the CLSI M44-A2 (21) document of Clinical and Laboratory Standards Institute (CLSI) with adaptations. Simvastatin (manipulated by SM Pharmaceutical LTDA) was activated by hydrolysis in 0.25 M NaOH at 35° C for 1 h and its pH was adjusted with HCl to 0.25 M, adapted from Lorenz & Parks (22). Fluconazole (Sigma-Aldrich) was prepared solution containing 5 mg/mL of dimethylsulfoxide (DMSO) at 10% following the manufacturer's instruction.

Firstly, the inoculum was adjusted to 10^7 cfu/mL, and 100 µL of solution were applied in plaques containing YPD agar (3% yeast extract, 1% peptone, 1% glucose, 2% agar). After that, 12.5, 25 and 50 µg of simvastatin and 50 µg of fluconazole were applied on paper disks, which were placed on the plaques. These were incubated at 35° C and after 24 h the the growth inhibition zone were calculated. *C. albicans* ATCC 90028 was considerate sensible to treatment with 25 µg of fluconazole with halo ranged from 28-39 mm.

Subsequently, another test was performed by placing two disks near each other, one containing simvastatin and the other containing fluconazole in order to ascertain the interaction of the drugs. 2.5 µg of fluconazole and 7.5 µg of simvastatin were used in this stage. The distance between these disks was calculated considering the size of the halos previously measured, according to following formula:

$$\text{Distance (mm)} = [\left(\frac{\text{Halo diameter (sim)}}{2}\right) + \left(\frac{\text{Halo diameter (flu)}}{2}\right) + \text{ disks diamete}] - 10$$

2.3. Antimicrobial susceptibility tests

Broth microdilution tests were made to determine the minimum inhibitory concentration (MIC) of the drugs in accordance with the document M27-S4 (23) with adaptations. According to the document, 96-well plaques with bottom "u" were filled with 100μL of RPMI-1640 medium (phenol red with glutamine and without bicarbonate) buffer plus MOPS [3- (N-morpholino) propanesulfonic acid] final concentration of 0.165 mol/L and pH 7.0. Then, 100 μL of drugs were placed through serial dilutions with fluconazole contractions ranging from 0.06-31.25μg/mL and simvastatin 0.24-125 μg/mL. The results were expressed as S (susceptible), SDD (susceptible dose-dependent) and R (resistant). The interpretation of the results to fluconazole was performed according to CLSI breakpoint which consider S ≤ 2 μg/mL, SDD = 4 μg/mL and R ≥ 8 μg/mL.

To evaluate the interaction of the drug was made according Checkerboard Technique (24) with fluconazole contractions ranging from 0.0075-31.25 μg/mL and simvastatin 0.12-31.25 μg/mL. Simvastatin was diluted vertically and fluconazole was then diluted on the horizontal. In each well was inoculated 10^3 CFU (colony forming units) and they were incubated for 48 h at 35° C and two visual readings were made at 24 and 48 h to determine the MIC 50. To categorize the interaction, the fractional inhibitory concentration (FIC) was calculated according to the equation (25): FIC = [fluconazole MIC (combination) / fluconazole MIC (alone)] + [simvastatin MIC (combination) / simvastatin MIC (alone)]. Result ≤ 0.5 = synergy,> 0.5 ≤ 4.0 = no interaction and > 4.0 = antagonism.

2.4. Survival assay

The survival assay was performed according to the methodology described by Li *et al.* (17). *G. mellonella* larvae were chosen in the final stage larvae, approximately 0.23 g, with cream color, without dark spots. They were inoculated by injection, in the last left proleg with $5x10^5$ CFU/larva into a suspension of 5 μL, and in the last proleg in the right side the antifungals were inoculated also in a suspension of 5 μL. In this step, was used a microsyringe Hamilton (701N, 26's gauge, 10μL capacity). 10 larvae per treatment and 5 for the control were used, and it was made in triplicate. Once infected, larvae were incubated at 37° C, kept

in the dark and monitoring was done by 10 days, on intervals of 24 h, in which the dead larvae were counted and discarded.

2.5. Statistical analysis

Standard deviation of all *in vitro* testing results was done. To compare the size of the halos of treatment with 25 μg of fluconazole and simvastatin were used the Student t test. This test was also used to compare the values of MIC of the drugs alone and in interaction. The results of the survival assay were analyzed by GraphPad Prism 6.0, were arranged in a survival curve using the Kaplan-Meier method and statistical analysis was performed by Log-rank test. For all tests the value of $p < 0.05$ was considered significant.

3. RESULTS

In disk diffusion test and broth microdilution test the *C. albicans* strain was sensitive to fluconazole. In disk diffusion test there was halo formation on treatment with simvastatin at all concentrations tested (Fig. 1). Inhibition halo was significantly higher in presence of 25 μg of fluconazole than that observed for sinvastatin. There was also a positive interaction between the drugs with 2.5 μg of fluconazole and 7.5 μg of simvastatin, as it is possible to see in Fig. 2 by the fusion of the halos. In broth microdilution test, simvastatin was also able to inhibit fungal growth and its combination with fluconazole has synergistic in 24 h according to the FIC (Table 1). The reduction of the concentration of the drugs placed alone and in interaction was statistically significant ($p < 0.05$).

The next step of this study was the survival test in *G. mellonella.* Some 80% of the larvae survived in 72 h when they received treatment with 50 mg/kg of fluconazole. The survival rate was low, about 40%, when a smaller dose of the drug was administered (12.5 mg/kg), which was observed at 120 h of infection. Given that there was no drug toxicity in these concentrations. As simvastin has never been tested in *G. mellonella,* a toxicity assay was made and it was observed that up to 500 mg/kg the drug was not toxic, however, at higher concentration (700 mg/kg) toxicity was observed (Fig. 3).

Subsequently, several *in vivo* infection assays were performed with simvastatin and there was no protective response (data not shown). Then, tests administering drugs in combination were made, one with 100 mg/kg of simvastatin and 10 mg/kg of fluconazole and

another with 700 mg/kg of simvastatin and 20 mg/kg of fluconazole, which resulted in the death of approximately 90% of the larvae at 150 and 100 h respectively, being statistically similar to the control group which received the inoculation with the fungus (Fig. 4).

4. DISCUSSION

In this work simvastatin was able to inhibit *C. albicans* growth in disk diffusion test, which is described for the first time, and microdilution broth, which already exists in the literature. An important question is that this strain showed trailing phenomenon in 48 h. Some isolates of *Candida* spp. may have residual growth phenomenon, which implies an overestimation of the MIC (26).

Synergy between statins and others antifungals has been studied for some years, authors showed that there is activity of the combinations made between statins and antifungal agents (27, 28). Other authors showed that there is synergy between statins and azoles against pathogenic fungus, including yeasts as *Candida* species, and molds as *Aspergillus* spp. and *Rhizopus* spp. (29, 30).

In the present study, the MIC of simvastatin against *C. albicans* was 31.25 μg/mL. This result was similar to Brilhante *et al.* (14) (29.45 μg/mL), four times lower than that was found by Menezes *et al*. (16) (128 μg/mL), and four times higher than the result of Nyilasi *et al.* (30) (8 μg/mL). Based on the result of the interaction by disk diffusion test, we suggest that there was a possible synergistic effect between fluconazole and simvastatin, which was confirmed by the microdilution broth test. Menezes *et al*. (16) and Brilhante *et al*. (15) showed the same result. Differently, Nyilasi *et al*. (30) found an additive interaction.

Currently, there is no experimental studies *in vivo* concerning the antifungal activity of simvastatin. Some authors have reported in clinical studies a decrease of *Candida* and zygomycetes isolates in patients with diabetes who were undergoing treatment with simvastatin (31, 32). However, Welch *et al.* (33) showed that there was no difference in the number of *Candida* isolates between patients who received or not received treatment with simvastatin, and no differences in mortality, length of stay and requiring intensive care.

The survival assay using larvae of *G. mellonella* was based on the study of Li *et al.* (19). They were the first to make the co-administration of two antifungal agents in *G. mellonella* larvae, tested the interaction of fluconazole and amphotericin B. They standardized inoculum of *C. albicans* at a concentration of $5x10^5$ cfu/larva and tested antifungal activity of

usual drugs in this host. They concluded that 90% of the larvae survived in 100 h of infection with treatment with 16 mg/kg of fluconazole. In our work 80% of the larvae survived in 72 h when they received 50 mg/kg of fluconazole, and with treatment with 12.5 mg/kg of this drug, 40% of then still alive in 120 h of infection.

There was no protective action of simvastatin *in vivo* in any concentrations tested, even in the highest concentration (700 mg/kg), in which the death rate was 50% at 120 h of infection. Simvastatin must have greatly inhibited the insect cholesterol synthesis which eventually led them to death. The reason is still unknown, the drug may be unable to penetrate the phagocytes or a larger amount of simvastatin would be required to inhibit the growth of these fungi. There was not a higher survival rates when the drugs were administered together, it may be due to the fact of fluconazole inhibits the enzyme such that it is responsible for the metabolism of simvastatin, the CYP3A4 (34).

Without being metabolized, simvastatin accumulates in the hemolymph and there is an increase of the adverse effects (30). It also acts by inhibiting the production of CoQ (Coenzyme Q: Ubiquinone) and heme A that are mevalonate subproducts (35). By inhibiting the synthesis of CoQ and heme A, simvastatin affects the oxygen reception and energy generation, impairing mitochondrial function (35). It ultimately causes myopathies that can lead to rhabdomyolysis (11). In humans, the incidence of myopathy in patients under treatment with standard doses of simvastatin is 0.02%. This percentage increases dramatically (60%) when this drug is associated with treatment with azole drugs (36-38)

Not always there is a correlation between antimicrobial activity *in vitro* and *in vivo*. For example, posaconazole, an antifungal used in the treatment of infections caused by *Mucor circinelloides* proved highly efficient *in vitro*, however, *in vivo*, its efficacy was poor (39). For the drug to be absorbed by the grastrointestinal tract, it must be recognized, dissolved and permeable. If any of these factors not to be attended, the activity of the drug will be reduced (40). The opposite can also happen when cannot activate the compound in the laboratory.

In conclusion, simvastatin showed great efficiency *in vitro* and its interaction with fluconazole was synergistic against *C. albicans*. However there was no correlation between its activity *in vivo*, with significant mortality of larvae of *G. mellonella*, when treated with the drug alone and in combination with fluconazole.

5. CONFLIT OF INTEREST

There were no conflicts of interest.

6. ACKNOWLEDGEMENTS

We are grateful to project's funding agency, National Council for Scientific and Technological Development (CNPq, Brazil), and the Higher Education Personnel Improvement Coordination (CAPES, Brazil) for providing the master's scholarships.

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8. CAPTIONS

Figure 1 - The measure of halos formed in disk diffusion susceptibility testing. Treatment with 25 μg of fluconazole (Flu) and 50, 25 and 12.5 μg of simvastatin (Sim). Mass given in μg.

Figure 2 - Positive interaction in the disk diffusion test, as is shown by fusion of the inhibition zones (black arrow). Disks with 16 mm distance, with 2.5 μg of fluconazole (left), and 7.5 μg of simvastatin (right).

Table 1 - Results of minimum inhibitory concentration (MIC) of fluconazole and simvastatin alone and in combination against *C. albicans* standard strain ATCC 90028 by reading after 24 h. The MIC values are expressed in μg/mL.

Figure 3 - *G. mellonella* survival curve. **A:** Infected with $5x10^5$ cfu of *C. albicans* ATCC 90028 and treated with 50 mg/kg and 12.5 mg/kg of fluconazole; **B:** Simvastatin toxicity screening, contractions of 700 mg/kg, 500 mg/kg and 100 mg/kg. Abbreviations: Sim = simvastatin, Flu = fluconazole, Ca = *C. albicans* ATCC 90028.

Figure 4 - *G. mellonella* survival curve infected with $5x10^5$ cfu of *C. albicans* ATCC 90028 and with concomitant use of the drugs. **A:** 100 mg/kg of simvastatin and 10 mg/kg of fluconazole; **B:** 700 mg/kg of simvastatin and 20 mg/kg of fluconazole. Abbreviations: Sim = simvastatin, Flu = fluconazole, Ca = *C. albicans* ATCC 90028.

Figure 1:

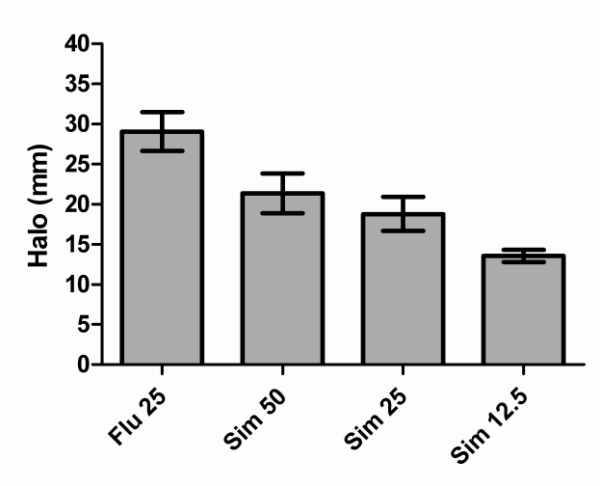

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Figure 2:

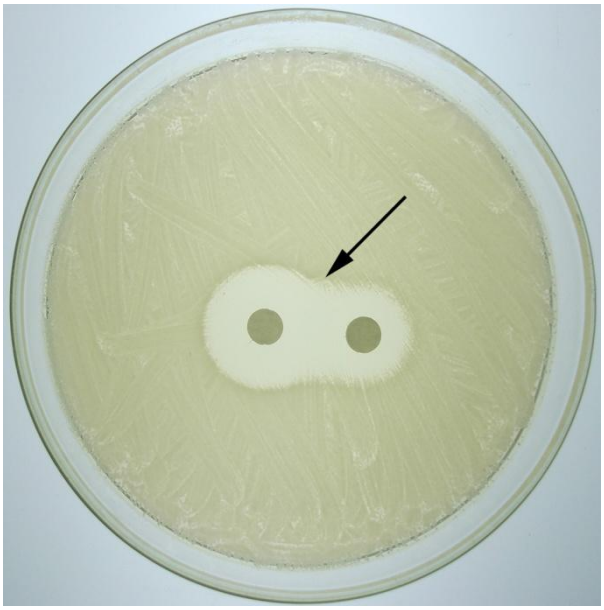

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Table 1:

Table 1 - Results of minimum inhibitory concentration (MIC) of fluconazole and simvastatin alone and in combination against *C. albicans* standard strain ATCC 90028 by reading after 24 h. The MIC values are expressed in μg/mL.

Strain	MIC Sim (alone)	MIC Sim (combination)	MIC Flu (alone)	MIC Flu (combination)	FIC	Int
C. albicans ATCC 22019	31.25	3.9	0.24	0.015	0.18	Syn

MIC: minimum inhibitory concentration, FIC: fractional inhibitory concentration, ATCC: American Type Culture Collection, Int: interaction, Syn: synergism, Sim: simvastatin, Flu: fluconazole.

Figure 3:

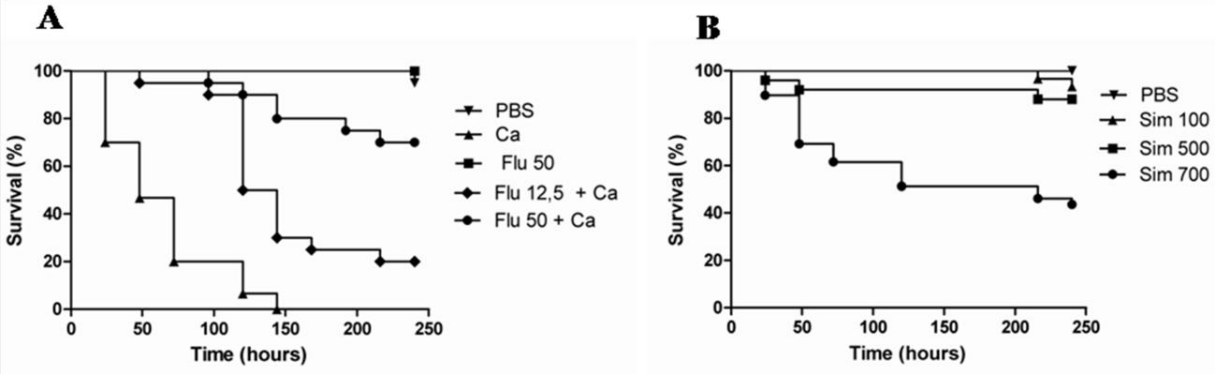

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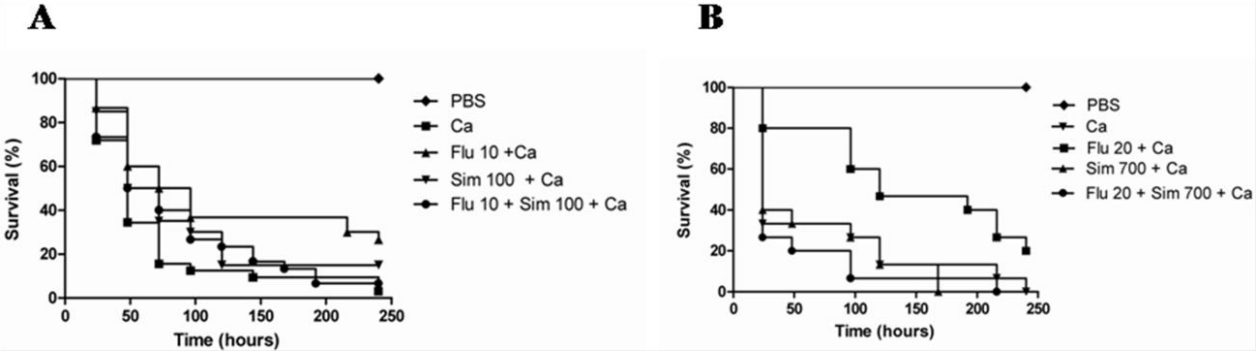
Figure 4:



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5.3. ARTIGO 3: Este artigo já se encontra publicado na revista Journal of Microbiological Methods.

Citação nas normas da revista:

De Souza, P.C., Morey, A.T., Castanheira, G.M., Bocate, K.P., Panagio, L.A., Ito, F.A., Furlaneto, M.C., Yamada-Ogatta, S.F., Costa, I.N., Mora-Montes, H.M., Almeida, R.S., 2015. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections. J. Microbiol. Methods 118, 182–186. doi:10.1016/j.mimet.2015.10.004

Citação nas normas da Associação Brasileira de Normas Técnicas (ABNT):

DE SOUZA, P. C. *et al*. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections. **Journal of microbiological methods**, v. 118, p. 182–186, 8 out. 2015.

REVISED

Tenebrio molitor (Coleoptera: Tenebrionidae) as an Alternative Host to Study Fungal Infections

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ABSTRACT

Models of host-pathogen interactions are crucial for the analysis of microbial pathogenesis. In this context, invertebrate hosts, including *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (nematode) and *Galleria mellonella* (moth), have been used to study the pathogenesis of fungi and bacteria. Each of these organisms offers distinct benefits in elucidating host-pathogen interactions. In this study, we present a new invertebrate infection model to study fungal infections: the *Tenebrio molitor* (beetle) larvae. Here we performed *T. molitor* larvae infection with one of two important fungal human pathogens, *Candida albicans* or *Cryptococcus neoformans*, and analyzed survival curves and larva infected tissues. We showed that increasing concentrations of inoculum of both fungi resulted in increased mortality rates, demonstrating the efficiency of the method to evaluate the virulence of pathogenic yeasts. Additionally, following 12 hours post-infection, *C. albicans* forms mycelia, spreading its hyphae through the larva tissue, whilst GMS stain enabled the visualization of *C. neoformans* yeast and their melanin capsule. These larvae are easier to cultivatein the laboratory than *G. mellonella* larvae, and offer the same benefits. Therefore, this insect model could be a useful alternative tool to screen clinical pathogenic yeast strains with distinct virulence traits or different mutant strains.

Keywords: *Candida albicans*; *Cryptococcus neoformans*; infection model; *Tenebrio molitor*

1. INTRODUCTION

The wax moth larvae (*Galleria mellonella*) have been usedin infectious disease research (Desalermos et al., 2012). Insects host have ethics, logistics and economic advantages over mammalian models (Li et al, 2013), and allow high efficiency testing on a large scale and at low cost (Lionakis, 2011). While USA has several commercial suppliers of *G. mellonella* larvae, Brazil has none, forcing researchers to maintain their own mothlarvae supply for the experiments. This increases costs of experiments and requires a person responsible for their maintenance.

An insect host on the rise is *Tenebrio molitor* (Coleoptera), a mealworm beetle, stored-grain plague (Schroeckenstein et al., 1990), whose larvae are used for feeding pets, as well as birds (Barker et al., 1998). The advantage of this alternative model is that *T. molitor* larva, as the moth larva, can be maintained at temperatures between 25 °C and 37 °C, what makes it suitable for the study of pathogens at body temperature (Li et al., 2013). It is an advantage compared to *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (nematode), whichdo not tolerate this temperaturerange (Desalermos et al., 2012). However, differently from *G. mellonella*, the mealworm caterpillars are commercialized in Brazil and are easier to cultivate in laboratory. Another advantage concerns the form of inoculation, whichis performedby injectionin the *T. molitor* model. On the other hand, in the *C. elegans* model, the pathogen is addedto the well where the nematode is prepared for the bioassay, resulting in a lack of accuracy regarding the number of microorganisms internalized by the host (Merkx-Jacques et al., 2013). Finally, its transcriptome was recently published (Oppert et al., 2012), offering opportunities for molecular biology advances in this field.

It is already known that *T. molitor* produces several antimicrobial peptides for defense against microbial pathogens, including fungi (Johnston et al., 2014). Different types of tenecin proteins are produced with antimicrobial activity against *Candida albicans* (Lee et al, 1999; Kim, 2001) and Gram-negative or Gram-positive bacteria (Chae et al., 2012; Rohet al., 2009). Furthermore, *T. molitor* larvae have been used to study *Staphylococcus aureus* and *Listeria monocytogenes* infections (Tindwa et al., 2013; Dorling et al., 2015). However, to this date, there are no reportsabout the use of the beetle's larvae as model host forhuman pathogenic fungi. Therefore, here we used *Cryptococcus neoformans* and *C. albicans* to study fungal pathogenesis in the *T. molitor* infection model system.

2. MATERIALS AND METHODS

2.1. Microorganisms and cultivation

Two reference strains were used in the experiments, *C. neoformans* ATCC 28957 and *C. albicans* SC5314, grown in Yeast Peptone Dextrose - YPD (1% yeast extract, 2% peptone, 2% glucose) at 37°C with agitation for 16 hours. Subsequently, the cells were centrifuged, the supernatant was discarded and the inoculum was washed 3 times with Phosphate Buffered Saline - PBS (137 mMNaCl, 2.7 mMKCl, 10 mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.4). Cell density was determined in a hemocytometer and suspensions with different cellular densities were producedwith PBS dilutions. Controls used heat-inactivated yeast at 65°C for 30 min. Loss of cell viability was determined by plating the suspensions on YPD agar and incubating for 48 hours at 37°C.

2.2. *T. molitor* larvae infection and survival curves

For the survival studies, we selected larvae weighing between 100-200 mg with clear, uniform color, without dark spots or grayish marks (Fig. 1A). We noticed that the uniformity of color and size ensures the reproducibility of experiments and prevents contamination of entomopathogens. The selected larvae were inoculated withthe pathogen of interest, using a Hamilton syringe (701N, 26's gauge, 10µL capacity). Inoculation was achieved by injecting theinoculum into the hemocoel, at the second or third visible sternite above the legs, in the ventral portion (Fig. 1B). Each larva of each group of 10 animals received 5µL of suspension containing the pathogen. Final concentrations are givenin figures when necessary. We used two control groups, one was inoculated with sterile PBS and the other with 10^6 heat-inactivated cells. The larvae were then incubated at 37 °C in Petri dishes containing rearing diet and the number of dead larvae was recordedon intervals of 24 hours during 10 days. To establish larvae death, we visually verified melanization and response to physical stimuli by gently touching them (Fig. 1C). The experiments were performed in triplicate with groups of ten animals, with a total of 30 larvae per group, which were used to build the survival curve. Results were analyzedwith GraphPad Prism 5, arranged in a survival

curve using the Kaplan-Meier method and statistical analysis was performed using log-rank test. P value < 0.05 was considered significant.

2.3. Microscopic analysis of infected tissue

To evaluate fungal infection within host tissue, three larvae were infected with 10^6 yeasts/5µL/larva and then incubated for 12 hours at 37°C. Chitin exoskelet on prevent fixative penetration in the larva and, thus tissue fixation. To circumvent this problem, animals were decapitated and the internal structures were embedded in liquid bacteriological agar 6 % (Kasvi) (Fig. 2 A and B). After solidification, the agar block was cut with a scalpel and fixed overnight in formaldehyde (Merck Millipore) 3.7% diluted in PBS. Dehydration, clearing and paraffin wax embedding of the fixed agar blocks was performed in an automated carrousel-type processor following an overnight processing schedule (Spencer and Bancroft 2008).

Slides with 5µm tissue sections were stained with Periodic Acid-Schifft (PAS) (Guarner and Brandt, 2011) for *C. albicans* infected larvae. Larvae infected with *C. neoformans* were stained with Gomori's Methenamine Silver (GMS) (Guarner and Brandt, 2011). Slides were analyzedby optical microscopy at 4,000 times magnification with a Leica DMI3000 light microscope.

3. RESULTS AND DISCUSSION

Invertebrate hosts, including *D. melanogaster*, *C. elegans* and *G. mellonella* have been used to study the pathogenesis of fungi and each of these organisms offers distinct benefits in elucidating host-pathogen interactions (Fuchs et al., 2010). To develop a new infection model system, we have evaluated different inoculum concentrationsof the fungi *C. albicans* and *C.neoformans*to infect *T. molitor*.

Regarding the negative controls, as shown in the Kaplan-Meier plots (Fig. 3A and B), all animals survived the injection procedures (PBS) and the inactivated microorganisms showed no toxicity to the caterpillars. These results indicate that live pathogenic fungi are necessary for lethality and that cellular components of both yeasts are not toxic for this host.

Increasing concentrations of inoculum of both fungi showed an increase in mortality rates, demonstrating the efficiency of the method to evaluate the virulence of pathogenic yeasts (Fig. 3 A and B). Moreover, the fungal load necessary to kill all 30 infected

larvae in 72 hours was much lower for *C. albicans* ($3x10^5$) (Fig. 3A) when compared to *C. neoformans* ($1x10^7$) (Fig. 3 B). However, whilst 10^4 *C. albicans* showed no virulence (no statistical difference from PBS, Fig. 3A), the same concentration of *C. neoformans* killed 50% of the infected animals (Fig. 3B). This difference was also shown in *G. mellonella* larvae infected with these fungi (Fuchs et al., 2010), suggesting that the fungi virulence behavior is similar in both insects.

In the present study, the established modelcan also be usedto observe differencesin fungal cell filamentationor capsule formation by *C. albicans* and *C. neoformans*, respectively. The last is known to forma melanin capsule within thehost during the infectionprocess and one of the main *C. albicans* virulence factors is the hyphal formation (Casadevall 2012; Wilson et al., 2009). Following 12 hours post-infection, as shown in Fig. 4 A, *C. albicans*forms mycelia, spreading its hyphae through the larva tissue (black arrows); immune cells migrate to the site of infection (red arrows); and yeast cells are observed (blue arrows). These histopathological findings are similar to those described in infected mice (Wilson et al., 2009), in haemoperfused liver from pig (Thewes et al., 2007), in the alternative chick embryochorioallantoic membrane model (Jacobsen et al., 2011), and in infected *G. mellonella* larva (Fuchs et al., 2010). Regarding *C. neoformans*, GMS stain enabled the visualization of yeast and their melanin capsule (white arrow, Fig. 4B), as seen in moth and mice models (Casadevall 2012; Fuchs et al., 2010). Thus, this insect model could be a useful alternative tool to screen clinical pathogenic yeast strains with distinct virulence traits or different mutant strains for further investigations.

Additionally, this model of infection represents an alternative for researchers from countries where *G. mellonella* is not commercially available. *T. molitor* larvae are also easierto raise and maintain, with nutritional requirements that can be met with regular diet (see Materials and methods 2.2), making it more suitable for cultivation in research laboratories.

4. CONCLUSIONS

In this study, we established the *T. molitor* infection model system to investigate fungal pathogenesis. It is the first report about the use of this beetle's larvae as model host for *C.neoformans* and *C. albicans*. To this date, there are only two reports using this infection system to study human pathogens (*S. aureus* and *L. monocytogenes*) (Tindwa et

al., 2013; Dorling et al., 2015). Thus, it isa valuabl emodel host but its full potential has yet to be reached. Although its transcriptome has been recently published, its genome has still not been sequenced. This molecular advance could allow the production of different *T. molitor* mutants, and contribute to studies on host response, as occurs with *C. elegans* and *D. melanogaster*. But even without thisinformation, much can be learned about fungal pathogens using *T. molitor* as amodel host.

5. ACKNOWLEDGEMENTS

Thisresearch was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), project 486042/2013-8.HMMM issuported by CONACyt (CB2011-166860). PCS and KPB are suportedby Master degreescholarshipsfrom Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil).

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Wilson, D., Thewes, S., Zakikhany, K., Fradin, C., Albrecht, A., Almeida, R., Brunke, S., Grosse, K., Martin, R., Mayer, F., Leonhardt, I., Schild, L., Seider, K., Skibbe, M., Slesiona, S., Waechtler, B., Jacobsen, I., Hube, B., 2009. Identifying infection-associated genes of

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7. LEGENDS

Figure 1 - *T. molitor* infection model system. A) Larvae weighing between 100-200 mg with clear, uniform color, without dark spots or grayish marks were selected. B) Inoculation was achieved by injecting theinoculum into the hemocoel, at the second or third visible sternite above the larva legs, in the ventral portion. C) Black arrows point dead larvae presenting their characteristic melanization.

Figure 2 - Procedures for histopathological preparation. Larvae were infected with 10^6 yeasts/larva and incubated for 12 h at 37°C. A) To collect internal structuresthe animals were decapitated. B) Tissues were introduced into liquid bacteriological agar 6%. C) After solidification, the agar blockwas cut with a scalpel. D) Fixation in formaldehyde 3.7% diluted in PBS.

Figure 3 - Survival curves of infected *T. molitor* with *C. albicans* (A) and *C. neoformans* (B). Kaplan-Meier survival plots show that increasing concentrations of inoculum of both fungi results in increased mortality rates, demonstrating the efficiency of the method to evaluate the virulence of pathogenic yeasts. Groups of 10 larvae were infected with the indicated fungal concentrations, repeated three times and pooled together to build survival curves containing 30 animals. Negative control groups where *T. molitor* larvae were injected with PBS or heat-inactivated fungi were included in the assay.

Figure 4 - Microscopic analysis of infected tissue. Larvae were infected with 10^6yeasts/5µL/larva and incubated for 12 h at 37 °C. The internal structures were fixed, embedded in paraffin and stained with PAS for *C. albicans* (A) and GMS for *C. neoformans* (B). Black arrows show hyphae spreading through the larva tissue. Red arrows show immune cells migrating to the site of infection. Blue arrows show yeast cells in the tissue. White arrow shows *C. neoformans* yeast.

Figure 1:

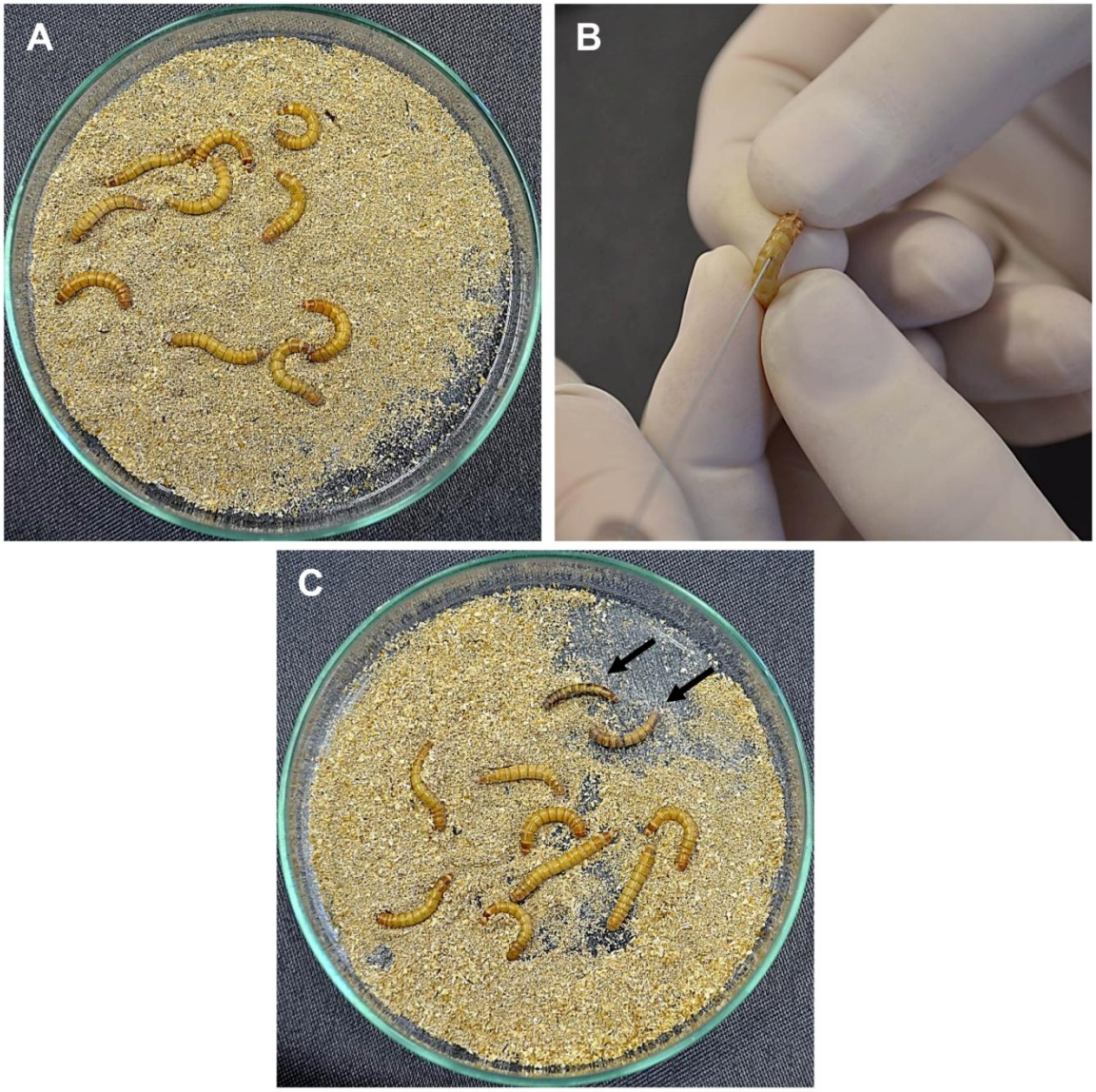

Fig. 1. *T. molitor* infection model system. A) Larvae weighing between 100-200 mg with clear, uniform color, without dark spots or grayish marks were selected. B) Inoculation was achieved by injecting theinoculum into the hemocoel, at the second or third visible sternite above the larva legs, in the ventral portion. C) Black arrows point dead larvae presenting their characteristic melanization.

Figure 2:

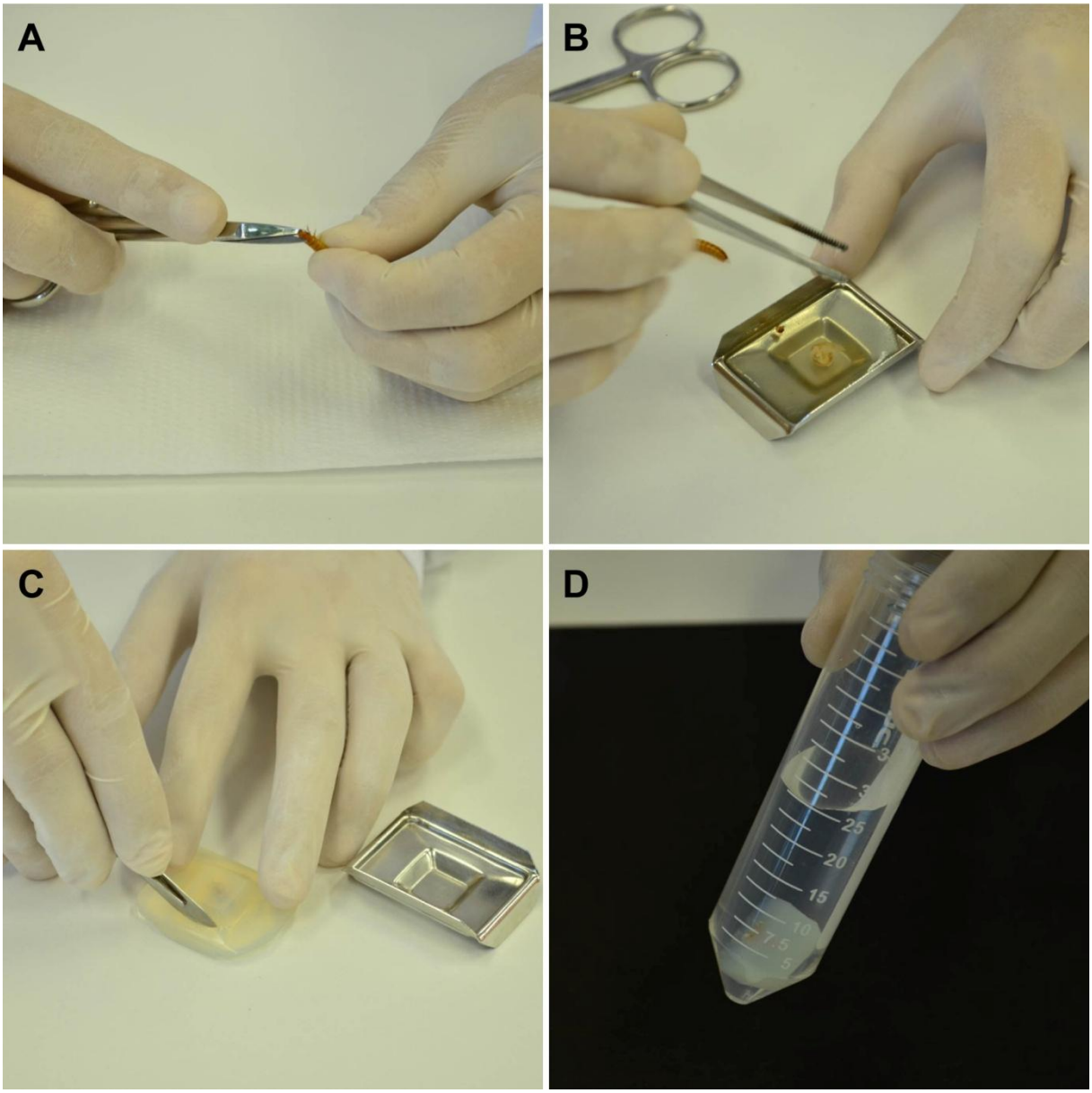

Fig. 2. Procedures for histopathological preparation. Larvae were infected with 10^6 yeasts/larva and incubated for 12 h at 37°C. A) To collect internal structures the animals were decapitated. B) Tissues were introduced into liquid bacteriological agar 6%. C) After solidification, the agar blockwas cut with a scalpel. D) Fixation in formaldehyde 3.7% diluted in PBS.

Figure 3:

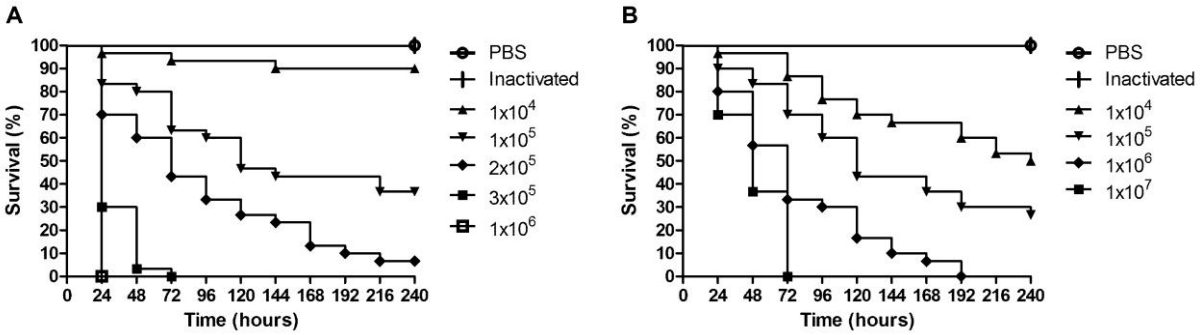

Fig. 3. Survival curves of infected *T. molitor* with *C. albicans* (A) and *C. neoformans* (B). Kaplan-Meier survival plots show that increasing concentrations of inoculum of both fungi results in increased mortality rates, demonstrating the efficiency of the method to evaluate the virulence of pathogenic yeasts. Groups of 10 larvae were infected with the indicated fungal concentrations, repeated three times and pooled together to build survival curves containing 30 animals. Negative control groups where *T. molitor* larvae were injected with PBS or heat-inactivated fungi were included in the assay.

Figure 4:

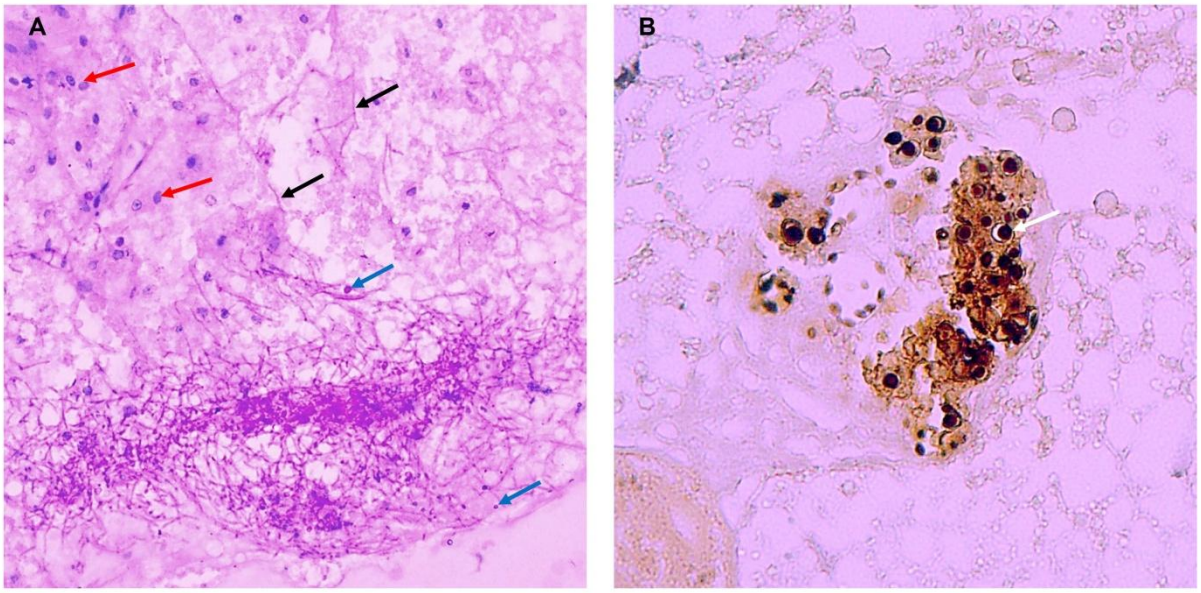

Fig. 4. Microscopic analysis of infected tissue. Larvae were infected with 10^6yeasts/5µL/larva and incubated for 12 h at 37 °C. The internal structures were fixed, embedded in paraffin and stained with PAS for *C. albicans* (A) and GMS for *C. neoformans* (B). Black arrows show hyphae spreading through the larva tissue. Red arrows show immune cells migrating to the site of infection. Blue arrows show yeast cells in the tissue. White arrow shows *C. neoformans* yeast.

6. CONCLUSÕES

Esse trabalho mostrou que a simvastatina foi capaz de inibir o crescimento *in vitro* de *C. albicans* e espécies raras de *Candida*. Foi necessária uma concentração dez vezes maior desse fármaco para inibir o desenvolvimento de *C. guilliermondii*, em relação à *C. lusitaniae.* Entretanto a sua interação com o fluconazol foi sinérgica para a primeira espécie e aditiva para a segunda. Esses experimentos mostraram que a co-administração dos dois fármacos reduziu a concentração necessária para inibir o crescimento das cepas.

Apesar dos resultados positivos *in vivo*, na experimentação*in viv*o com *C. albicans* sinvastatina não apresentou ação, nem quando a terapia foi combinada com fluconazol. A co-administração das drogas não foi eficaz talvez pelo fato do fluconazol inibir a CYP3A4, enzima responsável pela metabolização da sinvastatina, dessa forma aumentando seus efeitos adversos.

Esse trabalho também foi o primeiro a estabelecer o modelo de infecção de *T. molitor* para investigar patogênese fúngica de *C. neoformans* e *C. albicans*. Esse modelo invertebrado é importante, porém o seu potencial tem ainda de ser alcançado. Apesar da recente publicação de seu transcriptoma, o seu genoma ainda não foi sequenciado. Este avanço molecular poderia permitir a produção de diferentes mutantes de *T. molitor*, e contribuir para estudos sobre a resposta do hospedeiro. Entretanto, mesmo sem essa informação, muito pode ser aprendido sobre fungos patogênicos usando *T. molitor* como um modelo de infecção.