**UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO**

André Moreira Pessoni

**Avaliação da produção e composição de vesículas extracelulares de
*Aspergillus fumigatus***

**Ribeirão Preto
2021**

ANDRÉ MOREIRA PESSONI

**Avaliação da produção e composição de vesículas extracelulares de
*Aspergillus fumigatus***

Versão Corrigida

Dissertação apresentada ao
Departamento de Bioquímica da
Faculdade de Medicina de Ribeirão Preto
para obtenção do título de Mestre em
Ciências.

Área de Concentração: Bioquímica.

Orientador: Prof. Dr. Fausto Bruno dos
Reis Almeida.

Ribeirão Preto

2021

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FICHA CATALOGRÁFICA

Pessoni, André Moreira

Avaliação da produção e composição de vesículas extracelulares de *Aspergillus fumigatus* / André Moreira Pessoni; orientador, Fausto Bruno dos Reis Almeida. - 2021.

179 p.: Il.; 30 cm

Dissertação de Mestrado, Programa de Pós-Graduação em Bioquímica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Ribeirão Preto, 2021.

Versão original

1. *Aspergillus fumigatus*. 2. Vesículas extracelulares. 3. Metabólitos secundários. 4. Proteoma. 5. Espectrometria de massas. 6. Virulência.

Nome: PESSONI, André Moreira

Título: Avaliação da produção e composição de vesículas extracelulares de *Aspergillus fumigatus*.

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Ciências.

Área de Concentração: Bioquímica.

Aprovado em: ____/____/____

Banca Examinadora

Prof (a). Dr (a). _____ Instituição: _____

Julgamento: _____ Assinatura: _____

Prof (a). Dr (a). _____ Instituição: _____

Julgamento: _____ Assinatura: _____

Prof (a). Dr (a). _____ Instituição: _____

Julgamento: _____ Assinatura: _____

À minha família.
Especialmente à minha mãe, Taís,
que esteve ao meu lado em todos
os momentos, bons e ruins.

AGRADECIMENTOS

Ao meu orientador, **Dr. Fausto Bruno dos Reis Almeida**. Quando ele era doutorando e eu era calouro, ele me deu uma chance de trabalhar com ciência e acreditou em mim. A partir desse momento eu passei a acreditar também. Caminhamos juntos por muitos anos e por causa dessa parceria eu cresci, conheci pessoas maravilhosas e consegui atingir objetivos antes inimagináveis. Tenho a honra de ser seu amigo e de me inspirar com o seu exemplo. Agradeço imensamente por tudo, trabalhar com você é sempre fantástico.

À **Profa. Dra. Maria Cristina Roque Barreira**, por ter me dado a oportunidade de fazer parte do seu laboratório como aluno de iniciação científica e, depois, por disponibilizar a estrutura do laboratório para realização dos experimentos iniciais deste trabalho. No seu laboratório eu aprendi muito e percebi que gostava realmente de fazer ciência.

À **Profa. Dra. Silvana Guiliatti**, que, em um momento difícil da minha graduação, me mostrou o que é ser um bom bioinformata e se propôs a nos ensinar. Diferente de outros professores, ela nos tratou com humanidade e como colegas, nos mostrando o nosso valor. Sem os seus ensinamentos, talvez eu tivesse abandonado a graduação e não estaria escrevendo este texto hoje.

Ao **Prof. Dr. Gustavo Henrique Goldman**, por ceder a cepa que utilizei nesse estudo, pela disponibilização da estrutura do seu laboratório, pelas colaborações e por sempre confiar no nosso trabalho.

Aos **Prof. Dr. Roberto do Nascimento Silva**, **Prof. Dr. Rafael Silva Rocha** e à **Profa. Dra. Vânia Luiza Deperon Bonato**, pela disponibilização da estrutura dos seus laboratórios para realização de experimentos deste trabalho e pelas parcerias estabelecidas ao longo do tempo.

À **Profa. Dra. Taícia Pacheco Fill**, pela colaboração indispensável para a realização de uma parte deste trabalho, que elevou nossa proposta inicial.

Ao **Prof. Dr. Vitor Marcel Faça** e à **Msc. Ana Paula Masson**, pela disponibilização da estrutura do seu laboratório para realização de experimentos deste trabalho, em um momento de dificuldade, e pela gentil colaboração e ensinamentos compartilhados.

Ao **Carlos Alberto Vieira**, nosso técnico de laboratório. Obrigado por estar sempre disposto a embarcar nas nossas atividades, a compartilhar a sua experiência conosco, a aprender, a nos ajudar quando tudo está dando errado e a levar os quitutes mais deliciosos para mimar a gente.

À **Sandra Maria Oliveira Thomaz**, técnica de laboratório da Prof. Dra. Maria Cristina Roque Barreira. Obrigado por tudo o que você me ensinou, com toda paciência do mundo. Carrego o que aprendi na iniciação científica até hoje, isso me moldou e melhorou o meu trabalho como cientista.

À minha amiga e parceira de laboratório, **Dra. Verônica Soares Brauer**. Nós passamos por muitas coisas, sempre juntos, e você me ensinou demais. Obrigado por estar sempre disposta a me ouvir, a tirar minhas dúvidas e a ouvir meus palpites, e por me deixar a vontade para ter manias de organização. Estava inseguro quando comecei o mestrado, mas todas as comemorações, os choros, os cafés, as paçocas, as risadas e a parceria me deram força e me fazem uma pessoa melhor, sou muito grato.

À **Msc. Caroline Patini Rezende**, por toda amizade e convivência, desde quando ainda trabalhávamos na “masmorra” e quando tudo aqui era mato. Gosto de pensar que nós ajudamos o Fausto a iniciar o laboratório, mas sei que você foi mostrando o caminho e a gente foi ajudando a seguir.

Aos amigos do laboratório **Dra. Tamires Bitencourt**, que me ensinou muito e está sempre disposta a ajudar ou me levar para uma aula de muay thai; **Msc. Patrick dos Santos**, que me ouvia falar tanto do laboratório e decidiu ver por si mesmo, me sinto grato por ter você no nosso time; **Gabriel Trentim**, que sempre está pronto para animar o laboratório ou aceitar um convite para um churrasco; e **Bianca de Oliveira**

e **Msc. Mateus Freitas** por toda convivência e amizade. Obrigado a todos por acrescentarem tanto à nossas discussões científicas, às resoluções dos perrengues e ao bom funcionamento do laboratório.

Ao **Dr. Thiago Aparecido da Silva** e à **Msc. Patrícia Kellen Martins Oliveira Brito** por toda ajuda, ensinamentos e paciência, principalmente quando chegávamos ofegantes carregando nossas caixas com equipamentos ou precisando de um reagente.

Ao **Prof. Dr. Renato Graciano de Paula** e à **Dra. Mariana do Nascimento Costa**, por toda ajuda, compreensão e disponibilidade sempre.

Aos meus pais, **Taís Aparecida Moreira e Sérgio Henrique Pessoni**, pelo apoio e encorajamento sempre. Desde criança, sempre investiram na minha educação e isso é a maior herança que podem me dar. Sempre me incentivaram a ler livros e ver filmes, me acompanharam a teatros e shows, e me deram o suporte necessário para estudar. Quando optei por trancar um curso e prestar novamente o vestibular, vocês me apoiaram. Quando eu decidi fazer um curso “desconhecido”, vocês me apoiaram. Quando eu decidi seguir a carreira acadêmica, meio incerta para alguns, vocês me apoiaram. Não poderia oferecer outra coisa além de gratidão eterna.

À minha avó **Abadia Laura Moreira**, que me ensinou a falar, a cozinhar e a apreciar a música, minhas primeiras paixões (que me acompanham até hoje). Minha avó foi minhas pernas quando eu ainda não sabia andar, foi meu guia quando eu ainda não sabia para onde ir e cuidou de mim incontáveis noites em que eu fiquei doente. Ela, juntamente com meu avô **Manuel Rodrigues Moreira**, trabalharam incansavelmente para dar educação e conforto aos filhos e aos netos (e conseguiram!). Apesar de todas as dificuldades e preconceitos, eles lutaram e ela continua lutando pelo que acha certo.

À minha família, que, mesmo de longe, sempre me apoiou. Amo vocês!

Ao **Msc. Eduardo Domingos Borges**, que sempre está ao meu lado, para me ajudar com as burocracias da Universidade, para ouvir meus desabafos ou celebrar as nossas vitórias.

Aos **meus amigos**, que fiz durante o ensino médio, durante a faculdade (do meu e de outros cursos) e durante o mestrado. Eles sempre estiveram à disposição para me ajudar e me fizeram perceber que a gente não faz nada sozinho nessa vida. Seja com um bom papo, uma indicação de artigo ou uma bebida após o expediente, contribuíram imensamente para o meu crescimento.

À **Maria Ivone Campos Fonseca**, por ser um anjo na vida dos pós-graduandos. Mais do que uma funcionária exemplar, você é uma pessoa incrível, empática e prática. Obrigado por lidar tão bem com meu desespero ao me deparar com burocracias e sempre estar disposta a ajudar.

Aos **docentes, pós-graduandos e funcionários** da Faculdade de Medicina de Ribeirão Preto (FMRP), muito obrigado! A USP foi a minha segunda casa por quase 10 anos, muitos dos quais passei na FMRP, e sou muito grato por isso. Sem dúvidas, nunca esquecerei do meu tempo aqui. Agradeço a todos que contribuíram direta ou indiretamente para a conclusão deste estudo e para a minha formação como cientista.

Aos contribuintes do estado de São Paulo e do Brasil, que custearam minha educação, e aos governadores e presidentes que investiram em educação e ciência durante seus mandatos, permitindo à USP se tornar uma universidade de excelência e formar milhares de estudantes qualificados, impactando o país de forma imensurável. Somente com educação e ciência de qualidade construiremos um país melhor.

À **FAPESP** (Processo nº 2019/02504-2), **CAPES** e **CNPQ**, pelo apoio financeiro, indispensável para o desenvolvimento desse projeto.

Ninguém ignora tudo.
Ninguém sabe tudo.
Todos nós sabemos alguma coisa.
Todos nós ignoramos alguma coisa.

(FREIRE, 2017)

RESUMO

PESSONI, André Moreira. **Avaliação da produção e composição de vesículas extracelulares de *Aspergillus fumigatus***. 2021. 179 f. Dissertação (Mestrado) - Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2021.

Aspergillus fumigatus é considerado como o principal fungo causador de infecções em indivíduos imunocomprometidos, tendo grande importância médica. O sucesso das infecções depende da secreção de variadas moléculas ao espaço extracelular, mediadas, por exemplo, por meio de vesículas extracelulares (VEs). VEs fúngicas contêm proteínas, lipídios, pigmentos, polissacarídeos, DNA, RNA e outras moléculas que podem ser cruciais na fisiopatologia fúngica, mas existem poucos estudos que demonstrem a composição de VEs por fungos do gênero *Aspergillus*. Desta forma, nosso objetivo foi avaliar a produção e composição de VEs produzidas por *A. fumigatus* em meio líquido e sólido. Nossos resultados confirmam que *A. fumigatus* produz VEs em ambos os meios, com maior rendimento em meio sólido, e demonstram presença de metabólitos secundários, diversas populações de RNAs e proteínas. Entre as moléculas encontradas, pudemos identificar moléculas relacionadas à virulência fúngica, contra insetos, protozoários e células de mamíferos, à modulação da resposta imune de hospedeiros e, consequentemente, à sobrevivência do fungo. As descobertas relatadas neste estudo poderão ajudar a elucidar a função das VEs produzidas por *A. fumigatus* e entender os processos biológicos aos quais elas estão relacionadas.

Palavras-chave: *Aspergillus fumigatus*. Vesículas extracelulares. Metabólitos secundários. Proteoma. Espectrometria de massas. Virulência.

ABSTRACT

PESSONI, André Moreira. **Production and composition evaluation of extracellular vesicles from *Aspergillus fumigatus***. 2021. 179 f. Dissertação (Mestrado) - Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2021.

Aspergillus fumigatus is a fungus considered to be the main causative agent of infections in immunocompromised individuals, with great medical importance. The success of these infections depends on the secretion of several molecules to the extracellular space, mediated, for example, by extracellular vesicles (EVs). Fungal EVs contain proteins, lipids, pigments, polysaccharides, DNA, RNA and other molecules that can be crucial to fungal pathophysiology, however there are few studies demonstrating the composition of EVs produced by the *Aspergillus* genus. Thus, our objective was to evaluate the production and composition of EVs produced by *A. fumigatus* in liquid and solid media. Our results confirm that *A. fumigatus* produces EVs in both media, with higher yield in solid media, and demonstrate the presence of secondary metabolites, different populations of RNAs and proteins. Among the molecules found, we were able to identify molecules related to fungal virulence, with action against insects, protozoa and mammalian cells, to the modulation of host's immune response and, consequently, to the fungus survival. The findings reported in this study may help to elucidate the function of EVs produced by *A. fumigatus* and to understand the biological processes to which they are related.

Keywords: *Aspergillus fumigatus*. Extracellular vesicles. Secondary Metabolites. Proteome. Mass Spectrometry. Virulence.

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LISTA DE ABREVIATURAS E SIGLAS

µg – Micrograma

µl – Microlitro

1-DE – Eletroforese unidimensional

2-DE – Eletroforese bidimensional

A549 – Células epiteliais basais

alveolares adenocarcinômicas

humanas

ACAT2 – Acetil-CoA acetiltransferase

AIDS – Síndrome da imunodeficiência

adquirida

ATF4 – Fator de ativação da

transcrição 4

BCA – Ácido Bicinconínico

CCAAT – *motif* citosina-citosina-

adenosina-adenosina-timidina

COVID-19 – Doença do coronavírus

SARS-COV-2

DNA – Ácido desoxirribonucleico

DPPPA – *Deacetyl-pyripyropene A*

EBPβ – Proteína de ligação

potenciadora de CCAAT

eIF2α – Fator de iniciação eucariótico

2 alfa

EDTA – Ácido etilenodiamino tetra-

acético

ERK – Proteína quinase regulada por

sinal extracelular

FCFRP – Faculdade de Ciências

Farmacêuticas de Ribeirão Preto

FMRP – Faculdade de Medicina de

Ribeirão Preto

GNPS – *Global Natural Product Social*

Molecular Networking

HIV – Vírus da imunodeficiência

humana

M – Molar

m/z – Razão massa-carga

MAPK – Proteína quinase ativada por

mitógeno

mg – Miligramas

miRNA – Micro RNA

ML – Meio Líquido

mm – Milímetro

mM – Milimolar

mRNA – RNA mensageiro

MS – Meio Sólido

MS/MS – Espectrometria de massa em

tandem

ncRNA – RNA não-codificante

NF-κB – Fator nuclear kappa beta

nm – Nanômetro

NTA – Análise de rastreamento de

nanopartículas

p38 – Classe de MAPKs

PBS – Tampão fosfato-salino

PERK – Proteína quinase semelhante

a RNA do retículo endoplasmático

ppm – Partes por milhão

PPPA – *Pyripyropene A*

PPPQ – *Pyripyropene Q*

RIG-I – Gene I induzível por ácido

retinóico

RIN – Número de integridade do RNA

RIPA – Tampão de ensaio de
radioimunoprecipitação

RNA – Ácido Ribonucleico

RNA-Seq – Sequenciamento de RNA

rpm – Rotações por minuto

SDS – Dodecil sulfato de sódio

SDS-PAGE – Eletroforese em gel de
poliacrilamida com dodecil sulfato de
sódio

TNF- α – Fator de Necrose Tumoral
Alfa

tRNA – RNA transportador

USP – Universidade de São Paulo

VEs – Vesículas Extracelulares

VLDL – Lipoproteínas de muito baixa
densidade

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INTRODUÇÃO

1. INTRODUÇÃO

1.1. O gênero *Aspergillus*

Aspergillus é um gênero de fungos filamentosos, o qual inclui aproximadamente 200 espécies. São amplamente distribuídos no ambiente, com papéis importantes nos ecossistemas naturais e na economia, por seu potencial biotecnológico de produção de enzimas, ácidos orgânicos e metabólitos secundários importantes, e na saúde, visto que algumas espécies causam infecções nos vertebrados e, mais especificamente, em seres humanos (BENNETT, 2009; BENNETT, 2010; MOURA; CERQUEIRA; ALMEIDA, 2018; SZALEWSKI; HINRICHS; ZINNIEL; BARLETTA, 2018).

A morfologia do conidióforo, estrutura que carrega esporos assexuados, é o caráter mais clássico usado na taxonomia de *Aspergillus* e foi utilizada para nomear o gênero em 1729, mas frequentemente utiliza-se também a cor da colônia, a taxa de crescimento e a termotolerância para identificação da espécie (BENNETT, 2010; SUGUI; KWON-CHUNG; JUVVADI; LATGÉ *et al.*, 2014). No início do século XX, tornou-se um dos grupos de fungos mais conhecidos e estudados devido à sua prevalência no ambiente natural, facilidade de cultivo em laboratório e importância econômica (BENNETT, 2010), características relevantes que são complementadas pelos avanços da genômica, que permitiram o sequenciamento do genoma de diversas espécies, a criação e estudo de árvores filogenéticas mostrando relações evolutivas, e o surgimento de novas ferramentas para a avaliação desse gênero extremamente diverso (BENNETT, 2009).

Os fungos do gênero *Aspergillus*, os quais são saprófitos heterotróficos, crescem abundantemente na vegetação em decomposição e podem ser encontrados em feno mofado, solo, pilhas de composto orgânico e semelhantes (BENNETT, 2010). Seus esporos muito leves e de tamanho reduzido flutuam nas correntes de ar, dispersando-se em distâncias curtas e longas, dependendo das condições ambientais, além de serem carregados por insetos e animais (BENNETT, 2010; KANAANI; HARGREAVES; RISTOVSKI; MORAWSKA, 2008; KWON-CHUNG; SUGUI, 2013). Essa capacidade de se dispersar facilmente e crescer em diversas superfícies e tecidos com condições favoráveis possibilita usar o termo "onipresente" para descrever esses fungos (BENNETT, 2009).

1.2. *Aspergillus fumigatus*

Dentre as espécies patogênicas do gênero, podemos citar *Aspergillus fumigatus* como o principal causador de infecções em invertebrados (BENNETT, 2009) e o principal agente causador de infecções humanas em indivíduos imunocomprometidos, que têm doenças subjacentes (por exemplo HIV/AIDS, tuberculose, asma e cânceres) ou foram submetidos a procedimentos médicos que causam imunossupressão e eliminação de suas defesas (GIACOMAZZI; BAETHGEN; CARNEIRO; MILLINGTON *et al.*, 2016; MOURA; CERQUEIRA; ALMEIDA, 2018; WISEMAN, 2016). Essa infecção é denominada aspergilose e pode causar um espectro de doenças que variam de reações locais de hipersensibilidade a micoses sistêmicas, que são frequentemente fatais (GOMEZ; HACKETT; MOORE; KNIGHT *et al.*, 2010).

Essa infecção é facilitada pela inalação de centenas de conídios aerotransportados diariamente (LATGÉ, 1999; 2001) e, dessa forma, o patógeno é frequentemente encontrado em ambientes hospitalares (CHAZALET; DEBEAUPUIS; SARFATI; LORTHOLARY *et al.*, 1998; MAHIEU; DE DOOY; VAN LAER; JANSENS *et al.*, 2000; PINI; FAGGI; DONATO; SACCO *et al.*, 2008). Devido ao seu ciclo de vida saprófito, *A. fumigatus* apresenta majoritariamente fatores de virulência geralmente comuns também a outros fungos, como pigmentos e toxinas (por exemplo, melanina conidial e gliotoxina), que são parte do seu sistema de defesa e fornecem vantagens durante a infecção oportunista (SUGUI; KWON-CHUNG; JUVVADI; LATGÉ *et al.*, 2014). Essas moléculas imunomodulatórias produzidas por patógenos são geralmente encontradas na superfície das células, porém já foi demonstrado, em outros fungos, que essas moléculas também podem ser liberadas no espaço extracelular via VEs (KUIPERS; HOKKE; SMITS; NOLTE-'T HOEN, 2018). Além disso, o fungo apresenta produção de enzimas específicas, como catalases e superóxido dismutases, que o permitem sobreviver em ampla faixa de temperatura e pH, resistindo à estresse e possibilitando o crescimento do fungo em diversas condições (ABAD; FERNÁNDEZ-MOLINA; BIKANDI; RAMÍREZ *et al.*, 2010; KWON-CHUNG; SUGUI, 2013).

Recentemente, pacientes com pneumonia em decorrência da COVID-19 grave e síndrome do desconforto respiratório agudo podem se tornar propensos à

aspergilose pulmonar invasiva, a manifestação mais grave da doença causada por *Aspergillus*, devido à internação prolongada nas unidades hospitalares de tratamento intensivo, uso de esteroides e medicamentos imunomoduladores (ARASTEHFAR; CARVALHO; VAN DE VEERDONK; JENKS *et al.*, 2020; NASIR; FAROOQI; MAHMOOD; JABEEN, 2020). Dessa forma, a importância da elucidação dos mecanismos de infecção e virulência de *A. fumigatus* cresce ainda mais, visto que essas infecções conjuntas estão associadas a altas taxas de mortalidade e podem prolongar a fase aguda de COVID-19, aumentando o dano na saúde dos pacientes e no sistema de saúde.

1.3. Vesículas extracelulares

Vesículas extracelulares (VEs) são um conjunto de estruturas compostas por uma bicamada lipídico/proteica contendo variada carga, a qual inclui proteínas, lipídios, pigmentos, polissacarídeos, DNA e RNA (RODRIGUES; CASADEVALL, 2018; TKACH; THÉRY, 2016) e que expressam proteínas de superfície celular semelhantes às de sua origem e, portanto, podem aderir e se fundir, via endocitose ou fusão de membrana, a células próximas ou em circulação, entregando o seu conteúdo (ABELS; BREAKFIELD, 2016; KUBO, 2018). Podem ser secretadas por células de fungos, bactérias, mamíferos e outros organismos, sendo consideradas parte de um importante mecanismo conservado durante a evolução (BROWN; WOLF; PRADOS-ROSALES; CASADEVALL, 2015; TKACH; THÉRY, 2016), e são descritas desempenhando papéis de destaque na fisiologia e patogênese microbológica (BROWN; WOLF; PRADOS-ROSALES; CASADEVALL, 2015).

Embora o termo VEs se refira à todas as estruturas arredondadas e delimitadas por uma membrana de bicamada lipídico/proteica que transportam diferentes moléculas para o espaço extracelular, as VEs vêm sendo divididas em três categorias principais, as quais levam em conta a sua biogênese: exossomos, microvesículas e corpos apoptóticos (RODRIGUES; NAKAYASU; ALMEIDA; NIMRICHTER, 2014). Os exossomos possuem 30 a 100 nm de diâmetro e são oriundos da via endocítica; as microvesículas possuem de 100 a 1000 nm de diâmetro e resultam tanto de projeções (“brotos”) como de invaginações da membrana plasmática; e os corpos apoptóticos possuem de 1 a 4 µm, resultando da apoptose celular (ABELS; BREAKFIELD, 2016;

KUBO, 2018; TRICARICO; CLANCY; D'SOUZA-SCHOREY, 2017; VAN NIEL; D'ANGELO; RAPOSO, 2018). Devido ao surgimento de variadas classificações e com a finalidade de evitar diferentes nomenclaturas e minimizar erros de classificação, um novo modelo de definição foi sugerido pela Sociedade Internacional de Vesículas Extracelulares (ISEV). As diferentes populações de EVs, exceto os casos em que a biogênese seja demonstrada *in vivo*, são classificadas a partir do seu tamanho como pequenas (< 100 nm de diâmetro) e EVs médias ou largas (> 200 nm de diâmetro), ou de acordo com sua densidade (baixa, média ou alta) (THÉRY; WITWER; AIKAWA; ALCARAZ *et al.*, 2018).

Inicialmente, a secreção de VEs foi descrita como um meio de eliminar compostos desnecessários da célula. No entanto, sabemos que elas não transportam resíduos e tem um conteúdo que possibilita a troca de componentes entre células, atuando na sinalização durante processos homeostáticos celulares normais ou processos patológicos (ABELS; BREAKFIELD, 2016; VAN NIEL; D'ANGELO; RAPOSO, 2018).

1.3.1. Vesículas extracelulares fúngicas

As VEs produzidas por fungos foram primeiramente identificadas em *Cryptococcus neoformans* há mais de uma década, quando pesquisadores buscavam elucidar o funcionamento do transporte transmembrana de macromoléculas (RODRIGUES; NIMRICHTER; OLIVEIRA; FRASES *et al.*, 2007). Desde então, foram caracterizadas em diversos outros fungos, como *Aspergillus* spp. (BRAUER; PESSONI; BITENCOURT; DE PAULA *et al.*, 2020; SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019), *Candida albicans* (RODRIGUES; GODINHO; ZAMITH-MIRANDA; NIMRICHTER, 2015; VARGAS; ROCHA; OLIVEIRA; ALBUQUERQUE *et al.*, 2015; ZARNOWSKI; SANCHEZ; COVELLI; DOMINGUEZ *et al.*, 2018), *Cryptococcus* spp. (BIELSKA; SISQUELLA; ALDEIEG; BIRCH *et al.*, 2018; REIS; BORGES; JOZEFOWICZ; SENA *et al.*, 2019; RODRIGUES; GODINHO; ZAMITH-MIRANDA; NIMRICHTER, 2015; RODRIGUES; NAKAYASU; OLIVEIRA; NIMRICHTER *et al.*, 2008), *Histoplasma capsulatum* (ALBUQUERQUE; NAKAYASU; RODRIGUES; FRASES *et al.*, 2008), *Paracoccidioides brasiliensis* (DA SILVA; ROQUE-BARREIRA; CASADEVALL; ALMEIDA, 2016; VALLEJO; MATSUO;

GANIKO; MEDEIROS *et al.*, 2011), *Sporothrix brasiliensis* (IKEDA; DE ALMEIDA; JANNUZZI; CRONEMBERGER-ANDRADE *et al.*, 2018), *Trichophyton interdigitale* (BITENCOURT; REZENDE; QUARESEMIN; MORENO *et al.*, 2018) e outros, tendo seus componentes descritos como fatores de virulência, componentes imunologicamente ativos e diferentes macromoléculas indispensáveis aos mecanismos celulares (BROWN; WOLF; PRADOS-ROSALES; CASADEVALL, 2015; RODRIGUES; CASADEVALL, 2018; RODRIGUES; GODINHO; ZAMITH-MIRANDA; NIMRICHTER, 2015; RODRIGUES; NIMRICHTER; OLIVEIRA; FRASES *et al.*, 2007; VALLEJO; MATSUO; GANIKO; MEDEIROS *et al.*, 2011).

É sugerido que as VEs de diversos fungos têm a capacidade de desempenhar importantes mecanismos fisiopatológicos durante a infecção fúngica (BIELSKA; SISQUELLA; ALDEIEG; BIRCH *et al.*, 2018; BITENCOURT; REZENDE; QUARESEMIN; MORENO *et al.*, 2018; DA SILVA; ROQUE-BARREIRA; CASADEVALL; ALMEIDA, 2016; IKEDA; DE ALMEIDA; JANNUZZI; CRONEMBERGER-ANDRADE *et al.*, 2018; REALES-CALDERÓN; VAZ; MONTEOLIVA; MOLERO *et al.*, 2017; ZARNOWSKI; SANCHEZ; COVELLI; DOMINGUEZ *et al.*, 2018), bem como possivelmente podem atuar como veículos de comunicação entre espécies e de transferência de DNA ou proteínas que conferem resistência à antibióticos (BROWN; WOLF; PRADOS-ROSALES; CASADEVALL, 2015). Além disso, as VEs fúngicas, assim como as produzidas por bactérias (BROWN; WOLF; PRADOS-ROSALES; CASADEVALL, 2015), apresentam peculiaridades cujo mecanismo ainda não foi desvendado, por exemplo, como atravessam a parede celular para serem liberadas no espaço extracelular (JOFFE, L. S.; NIMRICHTER, L.; RODRIGUES, M. L.; DEL POETA, M., 2016).

1.4. Moléculas relacionadas à virulência

1.4.1. Metabólitos secundários

Uma característica marcante dos fungos filamentosos é a sua capacidade de produzir uma grande variedade de metabólitos secundários, compostos ativos naturais com baixo peso molecular que não desempenham nenhum papel no crescimento do organismo, mas estão envolvidos em sua relação com outros organismos, ajudando na adaptação e sobrevivência à diferentes ambientes e

aumentando a competitividade contra micro-organismos (GURUCEAGA; PEREZ-CUESTA; ABAD-DIAZ DE CERIO; GONZALEZ *et al.*, 2019; PAYNE; BROWN, 1998). Além de sua importância para a sobrevivência do fungo, os metabólitos secundários são de extrema importância para os humanos e seu impacto na sociedade tem sido tanto benéfico como maléfico.

Diversos metabólitos secundários são considerados fatores de patogenicidade e virulência por serem tóxicos aos hospedeiros (FALLON; REEVES; KAVANAGH, 2011; SUGUI; PARDO; CHANG; ZAREMBER *et al.*, 2007), causando dano celular e supressão da resposta imune por meio de micotoxicose (BENNETT; KLICH, 2003; FALLON; REEVES; KAVANAGH, 2011; PAYNE; BROWN, 1998; SUGUI; PARDO; CHANG; ZAREMBER *et al.*, 2007), além de causar prejuízos econômicos por contaminação de mercadorias agrícolas e prejuízos para a saúde pública (BENNETT; KLICH, 2003; PAYNE; BROWN, 1998).

Particularmente, o gênero *Aspergillus* é conhecido por ser grande produtor de produtos naturais bioativos, por produzir importantes micotoxinas, como gliotoxina, aflatoxinas, ocratoxina A e fumonisinas (KAMEI; WATANABE, 2005; KWON-CHUNG; SUGUI, 2009; PERRONE; GALLO, 2017), e também por produzir outras micotoxinas como citrinina, ácido ciclopiazônico, citocalasina E, fumagilina, fumitremorginas, ácido helvólico, patulina, ácido penicílico, esterigmatocistina e verruculogeno (GURUCEAGA; PEREZ-CUESTA; ABAD-DIAZ DE CERIO; GONZALEZ *et al.*, 2019; KAMEI; WATANABE, 2005; PERRONE; GALLO, 2017; REIJULA; TUOMI, 2003), representando vastas e potentes atividades biológicas. Por isso, um número considerável de pesquisas tem sido dedicadas para promover a identificação e quantificação de micotoxinas (BENNETT; KLICH, 2003), que podem estar associadas à interação ou comunicação dos fungos com outros organismos e o ambiente.

1.4.2. Ácido ribonucleico (RNA)

Uma composição diversa de material genético é encontrada em VEs. Em geral, carregam principalmente RNAs pequenos, além de RNAs mensageiros, microRNAs, RNAs não codificantes longos e curtos, fragmentos de RNAs transportadores, e outros tipos (ABELS; BREAKFIELD, 2016; BATAGOV; KUROCHKIN, 2013; DE PAULA; ANTONIÊTO; NOGUEIRA; RIBEIRO *et al.*, 2019). O empacotamento nas VEs permite

a proteção contra degradação no espaço extracelular e sua presença pode regular a expressão gênica, alterando diversos processos biológicos, como apoptose, desenvolvimento, diferenciação e proliferação celular, e regulação imunológica, ou servir como modelos para a produção de proteínas (ABELS; BREAKFIELD, 2016; HUNTZINGER; IZAURRALDE, 2011; KIM; ABDELMOHSEN; MUSTAPIC; KAPOGIANNIS *et al.*, 2017).

Estudos confirmam que VEs fúngicas carregam RNAs e que, como reportado anteriormente, essas moléculas estão relacionadas à fatores de virulência fúngica (JOFFE, LUNA S.; NIMRICHTER, LEONARDO; RODRIGUES, MARCIO L.; DEL POETA, MAURIZIO, 2016; PERES DA SILVA; PUCCIA; RODRIGUES; OLIVEIRA *et al.*, 2015) e, mais especificamente, ligados à processos de comunicação entre células, por meio de sinais para modulação da expressão gênica (IGUCHI; KOSAKA; OCHIYA, 2010; PERES DA SILVA; PUCCIA; RODRIGUES; OLIVEIRA *et al.*, 2015). Inclusive, pequenos RNAs foram apontados como fatores de resposta à infecção fúngica, modulando genes de virulência (CAI; QIAO; WANG; HE *et al.*, 2018), se mostrando importantes alvos de investigação.

1.4.3. Proteínas

As análises de proteínas encontradas em VEs de mamíferos são realizadas desde o fim do século XX, tendo grande importância para a definição de marcadores vesiculares. À medida que estudos sobre identificação das proteínas avançam, se tornou aparente que as VEs contêm uma subpopulação específica, ao invés de moléculas selecionadas aleatoriamente de suas células de origem ou desnecessárias e destinadas para eliminação (CHOI; KIM; KIM; GHO, 2015). Frequentemente são encontradas proteínas de choque térmico, do citoesqueleto, integrinas, enzimas, proteínas ribossomais (CHOI; KIM; KIM; GHO, 2015; DE PAULA; ANTONIÊTO; NOGUEIRA; RIBEIRO *et al.*, 2019) e estão comumente associadas aos mecanismos responsáveis pela biogênese, incluindo proteínas associadas à via endossômica, além de estarem envolvidas em transdução de sinal e apresentação de antígeno (ABELS; BREAKFIELD, 2016), importantes para a sobrevivência celular. Isso nos permite questionar se podem estar relacionadas também à virulência de alguns organismos.

A. fumigatus conta com diversos mecanismos para resistir às mudanças do ambiente ao aderir a um novo substrato ou organismo, processo que envolve diversas proteínas. O fungo deve ser capaz de obter nutrientes, resistir à mudança de temperaturas e evitar a morte induzida pelo sistema imunológico (LEIPHEIMER; BLOOM; PANEPINTO, 2019), então o processo inicial da infecção apresenta diversos fatores de estresse, por exemplo estresse oxidativo provocado por macrófagos ou neutrófilos (KNIEMEYER; LESSING; BRAKHAGE, 2009; SUGUI; KIM; ZAREMBER; CHANG *et al.*, 2008), gerando uma resposta diferencial na expressão genética do fungo, que busca se defender e permitir o estabelecimento ou prolongamento da infecção (SUGUI; KIM; ZAREMBER; CHANG *et al.*, 2008). Já foi reportado que proteínas encontradas na superfície conidial estão ligadas à adesão celular, minimização da identificação pelo sistema imune (VOLTERSEN; BLANGO; HERRMANN; SCHMIDT *et al.*, 2018) e modulação de quinases, podendo apresentar vantagens ao fungo (FULLER; RHODES, 2012), nos motivando a investigar em quais atividades as proteínas presentes nas VEs produzidas por *A. fumigatus* estarão envolvidas.

OBJETIVOS

2. OBJETIVOS

2.1. Objetivos gerais

Avaliar a produção e a composição de vesículas extracelulares produzidas por *A. fumigatus*.

2.2. Objetivos específicos

1. Avaliar a produção de VEs por *A. fumigatus* cultivado em meio líquido;
2. Avaliar a produção de VEs por *A. fumigatus* cultivado em meio sólido;
3. Caracterizar as VEs por *Nanoparticle Tracking Analysis* (análise de rastreamento de nanopartículas);
4. Investigar a composição das VEs, analisando:
 - 4.1. Metabólitos secundários;
 - 4.2. Proteínas;
 - 4.3. RNAs;
5. Analisar de forma integrada os dados obtidos, avaliando possíveis funções na virulência e sobrevivência fúngica.

MATERIAL E MÉTODOS

3. MATERIAL E MÉTODOS

3.1. Condições de cultivo

A cepa CEA17 de *Aspergillus fumigatus* utilizada neste estudo foi gentilmente cedida pelo Prof. Gustavo Henrique Goldman, da Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP) da Universidade de São Paulo (USP), Brasil. A cepa foi mantida a 37° C em meio com extrato de malte e ágar [extrato de malte 2% (p/v), glicose 2% (p/v), extrato de levedura 0,2% (p/v), solução de elementos traços (sulfato de zinco heptahidratado 75 mM, ácido bórico 180 mM, cloreto de manganês tetra hidratado 25 mM, sulfato de ferro heptahidratado 18 mM, cloreto de cobalto pentahidratado 6 mM, sulfato de cobre pentahidratado 6 mM, molibdato de amônio tetra hidratado 1 mM, EDTA 140 mM) 0,1% (v/v) e ágar 2% (p/v)] por 3 a 5 dias para esporulação.

Para todos os experimentos, foram preparadas suspensões de esporos em água pura estéril. Nos experimentos de padronização inicial em meio líquido (ML) e para análise da presença de metabólitos secundários nas VEs produzidas em ML, a suspensão de esporos foi utilizada para depositar 2×10^7 esporos em 400 ml de cultura líquida em meio mínimo [glicose 1% (p/v), solução de sais 20X (nitrato de sódio 1,41 M, cloreto de potássio 0,14 M, dihidrogenofosfato de potássio 0,22 M, sulfato de magnésio heptahidratado 0,042 M) 5% (v/v) e solução de elementos traços 0,1% (v/v), pH 6.5]. As culturas líquidas foram incubadas sob agitação a 200 rpm por 72h a 37° C.

Nos experimentos de padronização em meio sólido (MS) e para análise da presença de metabólitos secundários, proteínas e RNAs nas VEs produzidas em MS, a suspensão de esporos foi utilizada para depositar 1×10^7 esporos em cultura sólida em meio mínimo com ágar [glicose 1% (p/v), solução de sais 20X, solução de elementos traços 0,1% (v/v) e ágar 2% (p/v), pH 6.5]. As culturas sólidas foram incubadas a 37° C por 24h, 48h e 72h para a padronização e 48h para isolamento de VEs. Todos os experimentos foram realizados com três réplicas biológicas e, após o período de incubação, as culturas foram utilizadas para o processo de isolamento de VEs.

3.2. Isolamento de vesículas extracelulares

O isolamento das VEs obtidas de culturas em ML foi realizado como descrito para outros fungos (ALMEIDA; WOLF; DA SILVA; DELEON-RODRIGUEZ *et al.*, 2017; DA SILVA; ROQUE-BARREIRA; CASADEVALL; ALMEIDA, 2016; PERES DA SILVA; PUCCIA; RODRIGUES; OLIVEIRA *et al.*, 2015; RODRIGUES; NIMRICHTER; OLIVEIRA; FRASES *et al.*, 2007), com pequenas modificações. Após o período de incubação, a cultura foi filtrada por filtro Miracloth (Merck-Millipore, EUA) e o micélio recolhido foi congelado em nitrogênio líquido e armazenado à -80° C. O sobrenadante obtido foi concentrado aproximadamente 200 vezes por meio do sistema de ultrafiltração Amicon, utilizando membranas de celulose regenerada com *cutoff* de 100 kDa (Merck-Millipore, EUA). Células restantes foram removidas por centrifugação a 5,000 x g por 15 minutos a 4°C, e o sobrenadante coletado foi centrifugado a 15,000 x g por 15 minutos a 4°C para remover detritos. Posteriormente, o sobrenadante obtido foi submetido a ultracentrifugação a 100,000 x g por 1 h a 4°C para isolamento das VEs. Após o descarte do sobrenadante, os *pellets* foram ressuspensos em cerca de 300 µl de água estéril (Sigma-Aldrich, EUA) e mantidos a 4° C até etapa de quantificação. Após análise no sistema de rastreamento de nanopartículas, as VEs obtidas foram congeladas em nitrogênio líquido e liofilizadas.

O isolamento das VEs obtidas de culturas em MS foi realizado como descrito para *Cryptococcus gattii* (REIS; BORGES; JOZEFOWICZ; SENA *et al.*, 2019), com modificações. Após o período de incubação, as placas de petri com fungo foram lavadas com tampão fosfato-salino (PBS) estéril e o micélio foi recuperado com uma alça inoculatória. A solução obtida foi filtrada por filtro Miracloth (Merck-Millipore, EUA) e o micélio recolhido foi congelado em nitrogênio líquido e armazenado à -80° C. As células restantes na solução filtrada foram removidas por centrifugação a 5,000 x g por 15 minutos a 4°C e os detritos foram removidos por centrifugação a 15,000 x g por 15 minutos a 4°C. O sobrenadante obtido foi concentrado aproximadamente 50 vezes por meio do sistema de ultrafiltração Amicon, utilizando membranas de celulose regenerada com *cutoff* de 100 kDa (Merck-Millipore, EUA). Posteriormente, a solução concentrada foi submetida à centrifugação a 15,000 x g por 15 minutos a 4°C, o *pellet* foi descartado e o sobrenadante foi submetido à ultracentrifugação a 100,000 x g por 1 h a 4°C para isolamento das VEs. Após descarte do sobrenadante, o *pellet* foi ressuspenso em cerca de 300 µl de água estéril (Sigma-Aldrich, EUA) e mantido a 4° C até etapa de quantificação. Após análise no sistema de rastreamento de

nanopartículas, as VEs obtidas foram congeladas em nitrogênio líquido e liofilizadas (no caso dos experimentos para análise de metabólitos secundários) ou armazenadas à -80° C (para os demais experimentos).

3.3. Rastreamento de nanopartículas

A técnica *nanoparticle tracking analysis* (NTA, análise de rastreamento de nanopartículas) foi utilizada para caracterizar as preparações contendo as VEs fúngicas utilizando o aparelho NanoSight NS300 (Malvern Instruments, UK), equipado com captura rápida de vídeo e software para rastreamento de partículas, como descrito previamente (BITENCOURT; REZENDE; QUARESEMIN; MORENO *et al.*, 2018). As amostras utilizadas foram diluídas à aproximadamente 10^9 a 10^{10} partículas/ml e injetadas no sistema Nanosight, realizando três capturas de 30 segundos. A análise dos dados obtidos foi feita com o software NTA (Malvern Panalytical, versão 3.2.16).

3.4. Identificação de metabólitos secundários

Os metabólitos secundários presentes em um *pool* das VEs liofilizadas, com aproximadamente 1×10^{11} partículas/ml, foram extraídos com 200 µl de metanol e desidratados sob fluxo N₂. Os extratos foram diluídos em 100 µl de metanol, filtrados em filtros 0.22 em PTFE e analisados por espectrômetro de massa Thermo Scientific QExactive® Hybrid Quadrupole-Orbitrap. Análises foram realizadas no modo positivo com razão massa-carga (*m/z*) de 133-2000 Da, voltagem capilar de 3.4 kV, temperatura capilar de entrada de 280 °C e S-lens a 100 V. Uma coluna Thermo Scientific Accucore C18 2.6 µm (2.1 mm x 100 mm) foi utilizada como fase estacionária e foi injetado 5 µl da amostra. Para a fase móvel, foi utilizado ácido fórmico 0,1% (A) e acetonitrila (B). O perfil do eluente era (A/B): 95/5 até 2/98 dentro de 10 min, repouso por 5 min, até 95/5 em 1,2 min e repouso por 8,8 min. O tempo total de corrida foi de 25 min para cada corrida e a taxa de fluxo, 0.2 ml.min⁻¹ (COSTA; WASSANO; ANGOLINI; SCHERLACH *et al.*, 2019).

Os dados obtidos foram processados utilizando o software Xcalibur (versão 3.0.63), desenvolvido por Thermo Fisher Scientific. Os metabólitos foram identificados

por meio dos bancos de dados Dicionário de Produtos Naturais¹ e *Global Natural Product Social Molecular Networking* (GNPS). Para identificar metabólitos secundários em VEs de *A. fumigatus* usando o banco de dados GNPS, uma desreplicação das análises de MS/MS dos experimentos de 120h de incubação foi realizada usando o *workflow*² online no site GNPS³. Os dados foram filtrados removendo todos os íons de fragmento de MS/MS entre ± 17 Da do precursor m/z. Os espectros de MS/MS foram filtrados escolhendo apenas os 6 íons de fragmento superiores na janela de ± 50 Da em todo o espectro. A tolerância da massa do íon precursor foi estabelecida em 0,02 Da e a tolerância do íon do fragmento MS/MS em 0,02 Da. Os espectros foram então pesquisados nas bibliotecas espectrais do GNPS. Os espectros da biblioteca foram filtrados da mesma maneira que os dados de entrada. Todas as correspondências entre espectros selecionadas deveriam ter uma pontuação acima de 0,5 e pelo menos 5 picos combinados (WANG; CARVER; PHELAN; SANCHEZ *et al.*, 2016). Os resultados obtidos na base de dados GNPS estão disponíveis online⁴.

3.5. Extração de RNA

A extração de RNA foi feita, inicialmente, com dois protocolos distintos, com intuito de comparar rendimentos de RNA obtidos e a qualidade do material extraído. Foram utilizados o kit illustra RNAspin mini RNA isolation (GE Healthcare, EUA) e o kit miRNeasy mini (QIAGEN, Alemanha), mais eficiente para RNAs de tamanho menor.

Durante o processo de extração de RNA total de micélio, aproximadamente 30 mg do micélio foram macerados em nitrogênio líquido para total pulverização. Após, para as extrações realizadas com o kit illustra RNAspin mini RNA isolation, 350 μ l do tampão de lise LS e 3,5 μ l de β -mercaptoetanol (Sigma-Aldrich, EUA) foram adicionados ao micélio pulverizado, em um microtubo, e submetidos a vigorosa agitação. Nas extrações com o kit miRNeasy mini, cerca de 700 μ l de Qiazol (QIAGEN, Alemanha) foram adicionados ao micélio pulverizado, em um microtubo, e submetidos

¹ <http://dnp.chemnetbase.com>

² <https://ccms-ucsd.github.io/GNPSDocumentation/>

³ <http://gnps.ucsd.edu>

⁴ gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5d86fe698148480ca7836fcd59ff9458

a vigorosa agitação. Em seguida, o protocolo seguiu de acordo com as recomendações dos fabricantes.

Na extração de RNA total das VEs, as vesículas armazenadas à -80° C foram descongeladas e centrifugadas à 100.000 x g por 1h a 4° C para concentração do material. Nas extrações com o kit illustra RNAspin mini RNA isolation, o pellet obtido após ultracentrifugação foi ressuspenso em 350 µl do tampão de lise LS e 3,5 µl de β-mercaptoetanol (Sigma-Aldrich, EUA) e submetido a vigorosa agitação, com passos subsequentes de acordo com as recomendações do fabricante. Nas extrações realizadas com o kit miRNeasy mini, o pellet resultante foi ressuspenso em 275 µl de Qiazol (QIAGEN, Alemanha) e submetido a vigorosa agitação. Após, o protocolo seguiu de acordo com as recomendações dos fabricantes.

Para quantificação e análise de pureza do RNA obtido, as absorbâncias foram determinadas por aparelho NanoPhotometer™ Pearl (Implen, Alemanha), e para verificar a integridade dos RNAs extraídos, as amostras foram submetidas ao 2100 Bioanalyzer (Agilent, EUA) e analisadas com o 2100 Expert Software (Agilent, EUA). Além disso, as amostras de VEs também foram submetidas à quantificação fluorimétrica, método com maior precisão, no aparelho Qubit (Thermo Fisher Scientific, EUA).

3.6. Extração de proteínas

A extração de proteínas foi realizada como descrito previamente (SUBEDI; SCHNEIDER; PHILIPP; AZIMZADEH *et al.*, 2019), com pequenas alterações. Para extração das proteínas do micélio, aproximadamente 40 mg foram triturados em nitrogênio líquido até a consistência de pó. Em seguida, para o teste de extração, foi transferido para microtubos contendo um dos tampões: tampão de ensaio de radioimunoprecipitação (RIPA), Tris-SDS, Tris-Triton, PBS ou Laemmli [2% (p/p) dodecil sulfato de sódio (SDS), 10% glicerol, 60 mM Tris.HCl, pH 6.8], todos com 0,2% de coquetel inibidor de protease (Sigma-Aldrich, EUA), aquecidos a 95 °C por 5 minutos e depois armazenados a 4° C. Para a extração definitiva, o micélio pulverizado foi transferido para microtubos contendo tampão Tris-SDS com 0,2% de coquetel inibidor de protease (Sigma-Aldrich, EUA), aquecido a 95 °C por 5 minutos e armazenado a 4° C.

Na extração das proteínas das VEs, a quantidade total de VEs isoladas foi ultracentrifugada a 100,000 x g por 1 h a 4°C para concentração das amostras. Para o teste de extração, o sobrenadante foi descartado e o pellet foi ressuspensão em um dos tampões: RIPA, Tris-SDS, Tris-Triton, PBS ou Laemmli, todos com 0,2% de coquetel inibidor de protease (Sigma-Aldrich, EUA), aquecidos a 95 °C por 5 minutos e armazenados a 4° C. Para extração definitiva, o sobrenadante foi descartado e o pellet foi ressuspensão em tampão Tris-SDS com 0,2% de coquetel inibidor de protease (Sigma-Aldrich, EUA), aquecido a 95 °C por 5 minutos e armazenado a 4° C.

Todas as amostras, de micélio e VEs, foram sonicadas em amplitude 60%, pulso 10 *on* e 10 *off*, por um minuto. Posteriormente, os microtubos foram centrifugados a 14000 rpm por 10 minutos a 4° C e o sobrenadante foi transferido para novos microtubos. A quantificação das proteínas foi realizada, com diluição de 5 vezes, pelo método BCA [contendo solução de ácido bicinonínico e solução de sulfato de cobre (II) (Sigma-Aldrich, EUA)], segundo orientações do fabricante. As absorbâncias em 562 nm foram determinadas no leitor de microplacas ajustável VersaMax (Molecular Devices, EUA) para cálculo das concentrações por ml. Posteriormente, foi realizada eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE) 12%, corando o gel obtido com nitrato de prata, para evidenciar a presença de proteínas.

3.7. Preparo de amostras para identificação de proteínas

O método escolhido para o preparo das amostras foi o processo de preparo a partir de bandas de SDS-PAGE. Durante o preparo, foram utilizados 20 µl de cada uma das amostras de proteínas previamente extraídas de micélio e VEs produzidos por *A. fumigatus*, além de uma amostra com albumina de soro bovino (Thermo Scientific, EUA) como controle.

Em microtubos, foram adicionados 20 µl de proteína a ser tratada, 20 µl de tampão Tris-Ureia e 20 µl de DTT (10 µg/µl), e incubados com agitação de 350 rpm, por 30 minutos a 37° C. Após, foram adicionados 5 µl de tampão de amostra 5X sem 2-mercaptoetanol e os tubos foram fervidos a 100° C por 5 minutos. Com a amostra novamente em temperatura ambiente, foi adicionado iodocetamida (50 µg/µl) na proporção (1:5) e incubou-se por 30 minutos em temperatura ambiente e ao abrigo da

luz. Então, as amostras foram aplicadas no gel Mini-Protean TGX Precast Gel 4-20% (Biorad, EUA) e iniciou-se a corrida. Após o fim da corrida, o gel foi retirado do aparato e lavado em água ultrapura por 5 minutos, repetindo o processo 2 vezes, antes de corar o gel com solução de coomassie G-250 (Biorad, EUA), segundo recomendações do fabricante.

No dia seguinte, o gel foi enxaguado por 30 minutos em água ultrapura. Com o auxílio de uma placa de petri, régua e lâminas de bisturi descartáveis, as canaletas dos géis foram cortadas separadamente e cada uma foi dividida em seis pedaços iguais. Cada uma dessas seis partes foi dividida em outras quatro partes separadamente. Enquanto isso, microtubos devidamente identificados foram enxaguados com metanol 50% e as partes das canaletas foram transferidas para eles. Assim, realizou-se a etapa de descoração, adicionando 100 µl de acetonitrila/bicarbonato de amônio 100 mM (1:1) por 30 minutos a 37° C. Após o descarte do sobrenadante e a repetição destas etapas, as amostras foram armazenadas a 4° C.

No próximo dia, o sobrenadante foi retirado e foram adicionados 100 µl de acetonitrila, incubando por 10 minutos e depois descartando o solvente. O gel foi seco em uma concentradora à vácuo e foram adicionados 50 µl de tripsina (1 µg de tripsina diluída em bicarbonato de amônio 50 mM e 10% de acetonitrila) (Promega, EUA) a cada tubo, deixando em agitação de 350 rpm a 37° C até o dia seguinte.

Posteriormente, para interromper a atuação da tripsina, foram adicionados 5 µl de ácido fórmico a uma concentração de 10% a cada microtubo. Após, o sobrenadante foi transferido para um microtubo novo devidamente identificado e armazenado a 4° C. Ao gel que restou no microtubo antigo, foram adicionados 50 µl de acetonitrila 50% e ácido fórmico 5%, e incubou-se por 45 minutos. Ao fim da incubação, os microtubos com pedaços do gel foram levados para banho de ultrassom por 5 minutos e o sobrenadante foi transferido para o microtubo com sobrenadante coletado anteriormente. Este processo de incubação, banho de ultrassom e coleta de sobrenadante foi repetido mais uma vez. Ao gel que ainda restou no microtubo antigo, foram adicionados 30 µl de acetonitrila 90% e ácido fórmico 5%, e incubou-se por 5 minutos, transferindo o sobrenadante para o microtubo já utilizado anteriormente. Após, os microtubos foram secos em uma concentradora à vácuo e armazenados a 4° C.

Finalmente, o conteúdo dos microtubos foi obtido após ressuspensão com 10 µl de uma solução de acetonitrila 2% e passadas em *Stage Tips C18* como descrito previamente (RAPPSILBER; MANN; ISHIHAMA, 2007), para dessalinização. Ao fim, o conteúdo do tubo foi seco em uma concentradora à vácuo e enviado para espectrometria de massas.

3.8. Identificação de proteínas

Foi realizada espectrometria de massas Nano LC-MS, para amostras complexas, no equipamento q-ToF maxis 3G Bruker, que fornece alta resolução (60000 FWHM). Os dados brutos obtidos foram utilizados para uma busca com o mecanismo Mascot (PERKINS; PAPPIN; CREASY; COTTRELL, 1999), utilizando a base de dados “a_fumigatus 20181127”. Os parâmetros utilizados na busca foram: busca MS/MS *Ion Search*, modificações carbamidometil (C) fixa e oxidação (M) variável, massa de proteína irrestrita, tolerância de massa de peptídeo de ± 20 partes por milhão (ppm), tolerância de massa de fragmento de ± 0.6 Da e limiar de significância com $p < 0.05$.

Devido à alta resolução do equipamento, foram identificados diversos peptídeos com pequena variação de massa entre si, o que pode representar isótopos ou outras variações moleculares. Dessa forma, foi necessário a criação de um subconjunto utilizando como critério de seleção uma pontuação de significância para cada amostra. Foram excluídos os íons individuais com pontuação menor do que esse valor estabelecido para cada amostra pelo algoritmo do mecanismo Mascot. Após o corte, os valores de pontuação maiores que os limiares indicam proteínas com identidade ou homologia extensa com as encontradas na base de dados.

Posteriormente, bases de dados com informações de anotações genéticas de organismos catalogados como *Aspergillus fumigatus*, *Neosartorya fumigata*, *Aspergillus clavatus*, *Neosartorya fischeri* e *Emericella nidulans* foram obtidas de InterPro, UniProt, UniProtKB, Pfam e outros repositórios por meio da plataforma Quick GO (EMBL-EBI) (BINNS; DIMMER; HUNTLEY; BARRELL *et al.*, 2009). Somente com a união das bases de dados foi possível encontrar anotações sobre todas as proteínas identificadas na busca Mascot. Os arquivos com anotações foram importados para o R, ambiente para computação estatística, e utilizados para obter informações de

ontologia genética, como atividade desempenhada pela molécula e localização celular. As análises dos dados foram realizadas com código desenvolvido pelo autor e os gráficos foram gerados com o pacote ggplot2 (WICKHAM, 2016) e alterados com o software Photoshop, quando necessário.

RESULTADOS

4. RESULTADOS

4.1. Produção de VEs em meio sólido

A avaliação da produção de VEs por *A. fumigatus* em MS foi realizada após culturas por 24h, 48h e 72h, e isolamento das VEs produzidas. Após 24h, não foi observado crescimento fúngico suficiente para análise. Após 48h, a produção de VEs foi identificada e o tamanho da população obtida variou entre 20 e 240 nm (Figura 1A), com tamanho médio de 118,4 nm ($\pm 17,6$ nm), moda de 89,5 nm e produção de $2,30 \times 10^{12}$ ($\pm 3,74 \times 10^{11}$) partículas/ml, e após 72h, o tamanho das VEs obtidas variou entre 10 e 340 nm (Figura 1B), com tamanho médio de 156,8 nm ($\pm 5,1$ nm), moda de 128,9 nm e produção de $2,16 \times 10^{12}$ ($\pm 6,32 \times 10^{11}$) partículas/ml.

Figura 1. VEs produzidas em meio sólido analisadas por NTA.

Fonte: Produção do autor. VEs foram purificadas de culturas de *A. fumigatus* em MS e quantificadas por NanoSight NS300. Gráficos com a distribuição da concentração de

partículas/ml pelo tamanho em nanômetros, após (A) culturas de 48h e (B) após cultura de 72h. (C) Captura de tela obtida pelo aparelho NanoSight NS300 (Malvern Instruments, UK) mostrando a distribuição das VEs obtidas após cultura de 48h.

4.2. VEs produzidas por *A. fumigatus*

VEs produzidas por *A. fumigatus* foram isoladas e caracterizadas usando análise de rastreamento de nanopartículas para determinação do tamanho e perfil de distribuição. Nos experimentos de 72h em ML, o tamanho das VEs variou entre 20 e 500 nm (Figura 2A), com tamanho médio de 139,3 nm ($\pm 21,7$ nm), moda de 93,5 nm e produção de $2,25 \times 10^{10}$ ($\pm 2,37 \times 10^9$) partículas/ml. Nos experimentos de 120h, verificou-se em VEs obtidas a partir dos experimentos em ML, uma faixa de tamanho entre 50 e 850 nm (Figura 2B), com tamanho médio de 419,0 nm ($\pm 32,9$ nm), moda de 252,9 nm e produção de $3,07 \times 10^{10}$ ($\pm 5,92 \times 10^9$) partículas/ml, e verificou-se, em VEs obtidas a partir de experimentos MS, uma faixa de tamanho entre 50 e 400 nm (Figura 2C), com tamanho médio de 167,0 nm ($\pm 5,6$ nm), moda de 133,7 nm e produção de $6,59 \times 10^{10}$ ($\pm 2,20 \times 10^8$) partículas/ml, que indica uma produção cerca de duas vezes maior se comparada à produção de amostras de ML.

Figura 2. VEs produzidas por *A. fumigatus* analisadas por NTA.

Fonte: Produção do autor. VEs foram purificadas de culturas de *A. fumigatus* e caracterizadas por análise de rastreamento de nanopartículas usando o aparelho NanoSight NS300. Distribuição da concentração (partículas/ml) por tamanho em nanômetros de (A) culturas de 72h em ML, (B) culturas de 120h em ML e (C) culturas de 120h em MS.

4.3. Presença de metabólitos secundários

As VEs produzidas por *A. fumigatus* foram isoladas, o seu conteúdo de metabólitos secundários foi extraído e posteriormente caracterizado usando HPLC-

HRMS, tendo os resultados comparados com bancos de dados de produtos naturais. Com base na fórmula molecular, padrão de fragmentação MS/MS e correspondências com banco de dados GNPS, fomos capazes de identificar os metabólitos secundários presentes nos VEs. É importante notar que os metabólitos descritos foram detectados apenas na análise de VEs e não foram detectados nos experimentos de controle negativo (Figuras Suplementares S1-S6). Com base na análise de amostras de 72h em ML, três compostos foram identificados manualmente e os valores experimentais de m/z, fórmula de íons e erros (todos abaixo de 5 ppm) estão representados na Tabela 1. Em relação às amostras de 120h, cinco compostos foram identificados manualmente ou por meio do banco de dados GNPS (Figuras Suplementares S7-S10) e estão simultaneamente presentes em amostras de ML e MS. Destes compostos, dois são comuns às amostras de 72h e os valores experimentais de m/z, fórmula de íons e erros (todos abaixo de 5 ppm) são representados na Tabela 2. Todos os espectros de massas dos compostos identificados são relatados nos dados suplementares e suas estruturas moleculares são representadas na Figura 3.

Tabela 1. Metabólitos secundários identificados por espectrometria de massas em VEs produzidas por *A. fumigatus* em ML (72h).

Molécula	Fórmula [M+H] ⁺	m/z calculado	m/z experimental	Erro (ppm)
Pyripyropene A	C ₃₁ H ₃₇ NO ₁₀	584.2495	584.2492	-0.5
Pyripyropene Q	C ₃₀ H ₃₈ NO ₈	540.2597	540.2598	0.2
Fumitremorgin C	C ₂₂ H ₂₆ N ₃ O ₃	380.1974	380.1970	-1.1

Fonte: Produção do autor.

Tabela 2. Metabólitos secundários identificados por espectrometria de massas em VEs produzidas por *A. fumigatus* em ML e MS (120h).

Molécula	Fórmula [M+H] ⁺ ou [M-H ₂ O+H] ⁺	m/z calculado	m/z experimental	Erro (ppm)
Pyripyropene A	C ₃₁ H ₃₇ NO ₁₀	584.2495	584.2491	-0.7
Deacetyl-Pyripyropene A	C ₂₉ H ₃₅ NO ₉	542.2390	542.2388	-0.4
Fumiquinazoline C	C ₂₄ H ₂₀ N ₅ O ₃	426.1566	426.1558	-1.9

Fumitremorgin C	C ₂₂ H ₂₆ N ₃ O ₃	380.1974	380.1968	-1.6
Peroxyergosterol	C ₂₈ H ₄₄ O ₃	429.3368	429.3364	-0.9

Fonte: Produção do autor.

Figura 3. Estruturas moleculares dos metabólitos secundários.

Fonte: Produção do autor. Metabólitos secundários identificados em VEs produzidas por *A. fumigatus*: pyripyropene A, deacetyl-pyripyropene A, pyripyropene Q, peroxyergosterol, fumiquinazoline C e fumitremorgin C.

Dentre os metabólitos secundários encontrados, nas amostras de 72h em ML identificamos dois componentes de uma classe meroterpenóide, *pyripyropene A* (PPPA) e *pyripyropene Q* (PPPQ), e uma molécula alcaloide micotóxica, *fumitremorgin C*. Nas amostras de 120h, cultivadas em ML e MS, também identificamos dois componentes de uma classe meroterpenóide, PPPA e *deacetyl-pyripyropene A* (DPPPA). Ademais, identificamos um composto imidazol, *fumiquinazoline C*, e um esteroide, *peroxyergosterol*, além de *fumitremorgin C*.

A análise MS/MS do íon [M+H]⁺ em *m/z* 584.2492 produziu fragmentos em *m/z* 106.0292, 148.0395 (fragmento principal), 202.0500, 265.1227 e 318.1141 (Figura S1D) que foi putativamente identificado no banco de dados GNPS como PPPA (Figura S7). Em estudo anterior, a análise de fragmentação de um padrão de PPPA gerou fragmentos em *m/z* 106, 148 (fragmento principal), 202, 265 e 318 (LEE; CHAE; KIM;

CHAE *et al.*, 2019), o que permitiu uma identificação putativa do íon $[M+H]^+$ em m/z 584.2492 como PPPA. Para o íon $[M+H]^+$ em m/z 540.2598, a fórmula molecular obtida através da massa exata e da observação do PPPA nas VEs permitiu a identificação putativa de PPPQ, um metabólito secundário já conhecido por ser produzido por *A. fumigatus* (TOMODA; TABATA; YANG; NAMATAME *et al.*, 1996). No entanto, o espectro MS/MS do íon $[M+H]^+$ em m/z 542.2388, mostrou um padrão de fragmentação semelhante ao PPPA, com fragmentos em m/z 106.0291, 148.0382 (fragmento principal), 202.0498, 265.124, 288.6359 e 429.6415, e foi identificado como DPPPQ por meio do banco de dados GNPS (Figura S8). A análise MS/MS do íon $[M+H]^+$ em m/z 380.1970 produziu fragmentos em m/z 160.0757, 212.1072, 226.1228 (fragmento principal), 296.1397 e 324.1343. O mesmo padrão de fragmentação foi observado por outros autores para *fumitremorgin C* (FORNAL; PARFIENIUK; CZECHKO; BILINSKA-WIELGUS *et al.*, 2017), o que permitiu a identificação putativa do íon $[M+H]^+$ em m/z 380.1970 como essa micotoxina. O íon $[M-H_2O+H]^+$ em m/z 426.1558 foi putativamente identificado no banco de dados GNPS como *fumiquinazoline C* (Figura S9), um metabólito secundário já relatado para *A. fumigatus* (LIM; AMES; WALSH; KELLER, 2014). O padrão de fragmentação para o íon $[M-H_2O+H]^+$ em m/z 426.1558 exibiu íons em m/z 144.0809, 171.0916, 199.0865 (fragmento principal), 228.0767 e 240.0768. Um estudo anterior relatou uma fragmentação semelhante para a *fumiquinazoline C* obtida de *A. fumigatus*, com íons em m/z 171, 199 (fragmento principal), 228 e 373 (GAUTHIER; WANG; SIFUENTES DOS SANTOS; FYSIKOPOULOS *et al.*, 2012). Análise MS/MS do íon $[M+H]^+$ em m/z 429.3364 produziu fragmentos em m/z 69.0704 (fragmento principal), 83.0860, 135.0804, 191.1067, 393.3167 e 411.3248, que permitiu identificação putativa como *peroxygosterol* no banco de dados GNPS (Figura S10). Este esteroide foi isolado pela primeira vez de uma fonte biológica em *A. fumigatus* (CHEN; YUAN; BUCHANAN; QUEK, 2020) e fragmentação semelhante foi relatada por outros autores, com íons em m/z 69.07, 191.11, 393.32 e 411.32 (NIELSON; FREDRICKSON; WALLER; RENDÓN *et al.*, 2017).

4.4. Presença de RNA

Após a extração de RNA das VEs e micélios cultivados em MS por 48h, foram mensuradas as absorbâncias do material obtido, como pode ser visto na Tabela 3. As razões 260/280 obtidas, que representam a pureza do RNA (que absorve comprimentos de onda de 260 nm) e a ausência de contaminantes (que absorvem comprimentos de onda de 280 nm), estão próximas do valor ideal (razão 260/280 aproximadamente 2) e as razões 260/230, que indicam contaminantes que absorvem comprimentos de onda de 230 nm, estão abaixo dos valores esperados de aproximadamente 2, podendo sugerir a contaminação por carboidratos, produzidos em grande quantidade por *A. fumigatus* (GASTEBOIS; CLAVAUD; AIMANIANDA; LATGÉ, 2009), ou fenóis.

Tabela 3. Relações das absorbâncias e concentração obtidas no aparelho NanoPhotometer Pearl (Implen, Alemanha) relacionadas à qualidade dos RNAs obtidos de micélios e VEs seguindo o protocolo do kit ilustra RNAspin mini RNA isolation (GE Healthcare).

Amostras	Relação 260/280	Relação 260/230	Concentração ng/μl
VEs - Amostra 1	2,083	1,140	721
VEs - Amostra 2	2,050	1,012	158
VEs - Amostra 3	2,107	1,168	227
Micélio - Amostra 1	1,957	0,341	86,5
Micélio - Amostra 2	2,480	0,250	100
Micélio - Amostra 3	2,053	0,458	185

Fonte: Produção do autor.

Desta forma, houve a necessidade de investigar a integridade dos RNAs extraídos, como realizado previamente (DE PAULA; ANTONIÊTO; NOGUEIRA; RIBEIRO *et al.*, 2019; REIS; BORGES; JOZEFOWICZ; SENA *et al.*, 2019). Durante o processo, foi identificado que as VEs apresentam predominância de RNAs menores que 2000 nucleotídeos e ausência de RNAs ribossomais, como representado no gel virtual da Figura 4.

Figura 4. Perfil das amostras de RNA extraído das VEs e do micélio de *A. fumigatus*.

Fonte: Produção do autor. Perfil obtido pelo equipamento 2100 Bioanalyzer (Agilent, EUA). As canaletas de 1 a 3 são triplicata das amostras de micélios e as canaletas de 4 a 6 são triplicatas das amostras de VEs.

Avaliando os valores de RNA *integrity number* (RIN, número de integridade do RNA) na Figura 5, inferimos que as extrações de RNA realizadas a partir do micélio, mostraram-se dentro dos parâmetros esperados (RIN aproximadamente 7), apesar do valor não estar disponível para a amostra 1. Em relação às VEs, a ausência de RNAs ribossomais limita a determinação de nota de integridade, uma vez que o algoritmo utiliza os sinais de fluorescência e perfil deste tipo de RNA para a análise.

Figura 5. Análise do conteúdo de RNA.

Fonte: Produção do autor. Nos gráficos, o eixo x representa a altura de *threshold*, enquanto

o eixo y representa o tamanho. O primeiro pico apresentado em 25 nt refere-se ao controle da eletroforese microfluídica. Os gráficos na parte superior são triplicatas das amostras de micélios e os gráficos na parte inferior são triplicatas das amostras de VEs. Podemos observar a presença de RNAs pequenos nas VEs de *A. fumigatus*.

Após confirmar que as VEs produzidas por *A. fumigatus* contém RNA e que a integridade, assim como o perfil das amostras obtidas, estavam de acordo com o esperado, a extração de RNA das VEs e micélios cultivados em MS por 48h foi realizada com o outro kit, para verificação da melhora nas extrações. Devido às razões 260/230 mensuradas serem muito baixas para as amostras de micélio obtidas com o kit illustra RNAspin mini RNA isolation e a população de RNA identificada ter tamanho menor que 2000 nucleotídeos, o teste de extração de outras amostras com o kit miRNeasy mini foi realizado. Como pode ser visto na Tabela 4, os valores da razão 260/230 obtidos para amostras de micélio foram muito superiores e próximos ao valor esperado (razão aproximadamente 2), mostrando que o novo kit utilizado é superior nesse aspecto. Além disso, as razões obtidas para amostras de VEs se aproximam dos valores obtidos com o kit anterior, com a exceção da amostra 3, que apresenta uma relação 260/230 relativamente baixa, apesar da alta concentração.

Tabela 4. Relações das absorbâncias e concentração obtidas no aparelho NanoPhotometer™ Pearl (Implen, Alemanha) relacionadas à qualidade dos RNAs obtidos de micélios e VEs seguindo o protocolo do kit miRNeasy mini (QIAGEN).

Amostras	Relação 260/280	Relação 260/230	Concentração ng/μl
VEs - Amostra 1	2,010	0,858	167
VEs - Amostra 2	1,970	1,054	77,7
VEs - Amostra 3	1,790	0,347	88,4
Micélio - Amostra 1	2,024	1,930	160
Micélio - Amostra 2	2,077	1,932	376
Micélio - Amostra 3	2,280	2,082	737

Fonte: Produção do autor.

O kit miRNeasy mini (QIAGEN, Alemanha) foi selecionado como o método mais adequado para extração de RNA, devido às melhores relações gerais de qualidade.

Posteriormente, foi realizada uma quantificação mais precisa pelo fluorímetro Qubit (Thermo Fisher Scientific, EUA) (Tabela 5) para verificação das amostras obtidas de VEs produzidas por *A. fumigatus*. Os valores obtidos confirmam que a quantidade obtida após o protocolo seria suficiente para realização do sequenciamento de RNA (RNA-Seq) no futuro, visto que protocolos usualmente exigem no mínimo 10 ng/μl.

Tabela 5. Valores de quantificação fluorométrica obtidos no aparelho Qubit (Thermo Fisher Scientific) relacionados aos RNAs obtidos das VEs seguindo o protocolo do kit miRNeasy mini (QIAGEN).

Amostras	Concentração ng/μl
VEs - Amostra 1	21,6
VEs - Amostra 2	27,7
VEs - Amostra 3	33,2

Fonte: Produção do autor.

4.5. Presença de proteínas

Para seleção do método mais adequado de extração de proteínas, visto que os rendimentos de VEs isoladas e, conseqüentemente, de seu conteúdo proteico são reduzidos, foi realizado teste com cinco tampões: RIPA; Tris-SDS; Tris-Triton, como descrito anteriormente (SUBEDI; SCHNEIDER; PHILIPP; AZIMZADEH *et al.*, 2019); PBS; e Laemmli. Após a extração das proteínas, foram realizadas a quantificação e a eletroforese SDS-PAGE, para evidenciar sua presença no material obtido. O tampão Tris-SDS foi selecionado como método de extração, para amostras de VEs e micélio, por permitir a extração de quantidade satisfatória de proteínas, evidenciada por destacada visualização das bandas no gel de eletroforese na Figura 6.

Figura 6. Perfil eletroforético das VEs de *A. fumigatus*.

Fonte: Produção do autor. SDS-PAGE corado com nitrato de prata. Canaletas A, B, C, D e E contém as proteínas de VEs extraídas com os tampões RIPA, Tris-SDS, Tris-Triton, PBS e Laemmli, respectivamente.

Os valores de quantificação, na Tabela 6, foram utilizados como apoio na escolha do método. Apesar das quantidades obtidas nas amostras extraídas com tampão Laemmli ou Tris-Triton terem sido maiores que as outras, apresentam bandas muito discretas no gel e foram descartadas como possível método a ser utilizado, levando em consideração a sensibilidade do método e a possibilidade de incompatibilidade entre os reagentes utilizados, prejudicando o resultado.

Tabela 6. Concentrações das proteínas extraídas de VEs de *A. fumigatus* em teste de tampões, obtida após quantificação por BCA.

Canaleta	Tampão utilizado	ug/mL	Total (µg em 50 µl)
A	RIPA	99	4,95
B	Tris-SDS	89	4,45
C	Tris-Triton	124	6,2
D	PBS	24	1,2
E	Laemmli	424	21,2

Fonte: Produção do autor.

Desse modo, podemos verificar, pela quantificação representada na Tabela 6 e pelas proteínas marcadas na Figura 6, que as VEs de *A. fumigatus* contêm proteínas em seu interior e as carregam para o espaço extracelular.

4.5.1. Identificação de proteínas

Após extração das proteínas pelo método selecionado, a quantificação e preparação das amostras para espectrometria de massas foram realizadas. Na Tabela 7, está evidenciado que a concentração de proteínas varia entre amostras, provavelmente em decorrência de variabilidade biológica e do processo de extração, mas mantém uniformidade entre materiais da mesma amostra, apresentando valores crescentes da amostra 1 à amostra 3, para VEs e micélios.

Tabela 7. Concentrações das proteínas extraídas de VEs de *A. fumigatus*, obtidas após quantificação por BCA, utilizadas para protocolo de preparação para espectrometria de massas.

Amostras	Concentração ng/ml
VEs - Amostra 1	130
VEs - Amostra 2	1255
VEs - Amostra 3	3715
Micélio - Amostra 1	690
Micélio - Amostra 2	820
Micélio - Amostra 3	1230

Fonte: Produção do autor.

Após quantificação, as proteínas foram preparadas de acordo com o protocolo e submetidas à eletroforese SDS-PAGE. O perfil de tamanho das proteínas obtidas pode ser visto na Figura 7, que retrata as canaletas de gel com proteínas usadas no início do processo de preparo.

Figura 7. Perfil eletroforético das proteínas produzidas por *A. fumigatus*.

Fonte: Produção do autor. SDS-PAGE corado com coomassie. Canaletas A, B e C contém as proteínas obtidas de micélio, enquanto as canaletas D, E e F contém as proteínas obtidas de VEs.

Posteriormente, as proteínas preparadas foram enviadas para espectrometria de massas. As quantidades de proteínas obtidas em cada réplica independente ($n = 3$), após seleção do subconjunto de score adequado, podem ser vistas na Tabela 8.

Tabela 8. Quantidade de proteínas encontradas em cada amostra, após tratamento dos dados.

Amostras	Número de Proteínas
VEs - Amostra 1	161
VEs - Amostra 2	104
VEs - Amostra 3	37
Micélio - Amostra 1	24
Micélio - Amostra 2	53
Micélio - Amostra 3	154

Fonte: Produção do autor.

Utilizando as proteínas selecionadas, uma lista combinada de proteínas identificadas em todas as réplicas independentes ($n = 3$) foi gerada. A lista com os números de identificação (ID) na base de dados UniProtKB e a descrição de cada proteína pode ser vista na Tabela S1, para proteínas encontradas simultaneamente em amostras de micélio e VEs; na Tabela S2, para proteínas encontradas apenas em amostras de micélio; e na Tabela S3, para proteínas encontradas apenas em amostras de VEs. Das 398 proteínas identificadas, 180 foram identificadas em amostras de micélio, 218 foram identificadas em amostras de VEs e 70 foram identificadas tanto em amostras de micélio quanto em amostras de VEs.

Figura 8. Diagrama de Venn com as proteínas encontradas.

Fonte: Produção do autor. O diagrama representa o número de proteínas encontradas exclusivamente em uma das três condições: Encontradas somente em amostras de micélio (azul), encontradas simultaneamente em amostras de micélio e VEs (roxo) e encontradas somente em amostras de VEs (vermelho).

Foi realizada a análise qualitativa, fornecendo informações sobre atividades e localização celular das proteínas, por meio da plataforma Quick GO. Os resultados estão representados na Tabela 9, que contém informações sobre as atividades desempenhadas pelas proteínas identificadas em amostras de micélio e VEs, além de proteínas que são encontradas simultaneamente nas duas condições, e na Tabela 10, que contém informações sobre a localização de atuação das proteínas encontradas em amostras de micélio e VEs, além de indicar proteínas que compartilham a mesma localização nas duas condições. A Tabela 9 representa as funções celulares mais relevantes, sendo retratadas apenas as atividades que continham pelo menos 4

proteínas relacionadas. Ademais, todas as proteínas avaliadas estão relacionadas à, no mínimo, uma atividade e uma localização celular, podendo estar relacionadas a mais de uma atividade e mais de uma localização.

Tabela 9. Atividades desempenhadas pelas proteínas encontradas em amostras de micélio e VEs produzidas por *A. fumigatus*.

Atividade	Proteínas Micélio	Proteínas VEs	Proteínas em comum
Atividade de oxidorreductase	45	48	16
Constituinte estrutural do ribossomo	41	54	24
Ligação de ATP	35	40	11
Ligação de nucleotídeo	32	29	7
Ligação de íon metálico	28	37	9
Atividade de hidrolase	22	41	15
Ligação GTP	20	12	4
Ligação de RNA	19	19	8
Atividade de transferase	17	16	4
Atividade do fator de alongamento da tradução	11	9	-
Atividade catalítica	10	14	4
Atividade GTPase	10	8	3
Atividade da catalase	8	18	4
Ligação de DNA	8	7	5
Atividade de 5-metiltetrahydropteroyltryglutamato-homocisteína S-metiltransferase	6	-	-
Atividade de aminopeptidase	6	15	6
Atividade de gliceraldeído-3-fosfato desidrogenase (NAD ⁺)	6	-	-
Ligação de íon zinco	6	8	4
Atividade de isomerase	5	5	-
Ligação NAD	5	-	-

Ligação de ácido nucleico	5	4	-
Atividade de peptidase	5	18	5
Atividade da peroxidase	5	11	-
Atividade de fator de iniciação de tradução	5	-	-
Atividade 6-fosfogluconolactonase	4	-	-
Atividade dUTP difosfatase	4	-	-
Atividade da glutamato desidrogenase (NAD +)	4	-	-
Atividade de helicase	4	-	-
Ligação heme	4	10	-
Atividade de IMP desidrogenase	4	-	-
Ligação de íon magnésio	4	-	-
Atividade de metionina adenosiltransferase	4	-	-
Atividade do transportador de exportação de prótons tipo P	4	-	-
Atividade de peptidil-prolil cis-trans isomerase	4	5	-
Atividade de proteína dissulfeto isomerase	4	-	-
Atividade de heterodimerização de proteínas	4	-	-
Atividade de ATP sintase de transporte de prótons	4	4	-
Atividade da piruvato desidrogenase	4	0	-
Atividade de beta-glucosidase	-	8	-
Atividade de metalopeptidase	-	7	-
Atividade de aminoacil-tRNA ligase	-	6	-
Atividade de liase	-	6	-
Ligação de rRNA	-	6	-
Atividade de peptidase do tipo serina	-	6	-

Atividade da endopeptidase do tipo treonina	-	6	-
Atividade do transportador transmembrana acoplado a ATPase	-	5	-
Ligação de flavina adenina dinucleotídeo	-	5	-
Atividade do transportador transmembrana de prótons	-	5	-
Atividade ligase	-	4	-
Atividade de metaloexopeptidase	-	4	-
Atividade de N-acilsfingosina amidohidrolase	-	4	-
Atividade de fosfatidilserina descarboxilase	-	4	-
Atividade ATPase de transporte de prótons	-	4	-
Outros	84	96	-

Fonte: Produção do autor.

Tabela 10. Localização das proteínas encontradas em amostras de micélio e VEs produzidas por *A. fumigatus*.

Localização	Proteínas Micélio	Proteínas VEs	Proteínas em comum
Ribossomo	75	105	46
Membrana	37	74	16
Componente integral da membrana	24	55	11
Núcleo	22	41	8
Citoplasma	18	32	4
Membrana mitocondrial interna	9	6	4
Mitocôndria	9	5	2
Retículo endoplasmático	7	12	2
Região extracelular	7	7	2
Parede celular	5	1	1

Cromossomo	5	3	3
Membrana plasmática	5	-	-
Membrana de retículo endoplasmático	4	6	-
Vacúolo	3	4	3
Vesícula citoplasmática	2	-	-
Lúmen do retículo endoplasmático	2	2	1
Parede celular fúngica	2	-	-
Vesícula revestida de clatrina	1	-	-
Membrana mitocondrial externa	1	3	-
Grânulo de armazenamento de proteassoma	1	1	-
Respirassomo	1	-	-
Citosol	-	1	-
Endossomo	-	2	-
Membrana do endossomo	-	1	-
Aparelho de Golgi	-	2	-
Membrana de Golgi	-	1	-
Nucléolo	-	1	-
Local de quebra de fita dupla	-	1	-
Membrana vacuolar	-	1	-

Fonte: Produção do autor.

DISCUSSÃO

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5.1. Perfil das VEs obtidas

Os perfis das VEs produzidas em MS após 48h e 72h, obtidos por NTA, são similares entre si e similares ao perfil das VEs obtidas em ML. O perfil das VEs obtidas após 48h em MS mostra picos ao redor de 100 nm, 150 nm e 200 nm, assim como as VEs de ML (Figura 2), enquanto o perfil das VEs obtidas após 72h em MS apresentam um pico ao redor de 130 nm e outros picos menores em diversos tamanhos (Figura1). A abordagem em MS para isolamento de VEs de *A. fumigatus* é consideravelmente mais eficiente do que a abordagem em ML, com maior rendimento e protocolo mais rápido. Ademais, o padrão de VEs produzidas em MS foi semelhante ao obtido para a condição 72h em ML, que também está de acordo com análises de distribuição anteriores para *Aspergillus sp.* (BRAUER; PESSONI; BITENCOURT; DE PAULA *et al.*, 2020; RIZZO; CHAZE; MIRANDA; ROBERSON *et al.*, 2020; SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019).

5.2. Importância biológica dos metabólitos secundários encontrados em VEs produzidas por *A. fumigatus*

A. fumigatus é um importante patógeno com capacidade de produzir VEs (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019). Neste estudo, mostramos pela primeira vez que VEs produzidas por *A. fumigatus* contêm metabólitos secundários. Entre os metabólitos secundários identificados, PPPA foi previamente descrito como o primeiro inibidor altamente seletivo da isozima acil-CoA: colesterol aciltransferase 2 (ACAT2) obtido da cultura de *A. fumigatus* (MATSUDA; OHSHIRO; OHTAWA; YAMAZAKI *et al.*, 2015). Em células de mamíferos, ACAT2 desempenha um papel no fornecimento de colesterol esterificado para a formação de lipoproteína de densidade muito baixa (VLDL) e éster de colesterol para gotículas de lipídios citoplasmáticos, responsáveis pelo armazenamento de colesterol abundante presente no fígado, e aparece como um alvo terapêutico promissor em doenças como aterosclerose da artéria coronária (OHSHIRO; MATSUDA; SAKAI; DEGIROLAMO *et al.*, 2011; RUDEL; LEE; PARINI, 2005). Em fungos, ACAT2 é uma enzima importante na via de biossíntese do ergosterol, a contraparte do colesterol (SOTO; STRITZLER;

LISI; ALLEVA *et al.*, 2011). Ergosterol contribui para a integridade da membrana celular, sendo considerado como candidato para um potencial alvo antifúngico (ZHANG; WEI; FAN; JIN *et al.*, 2020). Além disso, o PPPA e seus derivados têm sido descritos como possíveis compostos inseticidas ecológicos, com atividade aficida (HORIKOSHI; GOTO; MITOMI; OYAMA *et al.*, 2017; 2018). Derivados de PPPA foram descritos atuando em proteínas relacionadas ao sistema nervoso de insetos e PPPA foi descrito como não apresentando toxicidade aguda contra ratos (HORIKOSHI; GOTO; MITOMI; OYAMA *et al.*, 2018; LEICHTER; THOMPSON; JOHNSON; SCOTT, 2013). PPPQ e outros derivados, como DPPPQ, também compartilham as propriedades inibitórias de ACAT2, mas em menor extensão (MATSUDA; OHSHIRO; OHTAWA; YAMAZAKI *et al.*, 2015; TOMODA; TABATA; YANG; NAMATAME *et al.*, 1996). Em suma, isso pode implicar que a presença dessas moléculas em VEs pode estar relacionada à interação do fungo com o metabolismo do hospedeiro ou, em outra perspectiva, atuando como moléculas fungitóxicas para outros microrganismos no microambiente do hospedeiro. Além disso, podem ser utilizadas como ferramenta de sobrevivência, protegendo os arredores contra a presença de insetos.

Em sequência, identificamos uma molécula do grupo das fumiquinazolin, que são conhecidas moléculas com atividades antibacteriana (SILVA; FURTADO; PUPO; FONSECA *et al.*, 2004), antifúngica (BELOFSKY; ANGUERA; JENSEN; FENICAL *et al.*, 2000) e antitumor (LONG; RESENDE; KIJJOA; SILVA *et al.*, 2019; RESENDE; BOONPOTHONG; SOUSA; KIJJOA *et al.*, 2019; TAKAHASHI; MATSUSHITA; DOI; MINOURA *et al.*, 1995). São relatadas como levemente citotóxicas, produzidas por mais de 40 isolados de *A. fumigatus* e comumente encontradas em esporos, com sua produção regulada e ligada à esporulação assexuada (FRISVAD; RANK; NIELSEN; LARSEN, 2009; GAUTHIER; WANG; SIFUENTES DOS SANTOS; FYSIKOPOULOS *et al.*, 2012; LIM; AMES; WALSH; KELLER, 2014). Há estudos mostrando menor produção de *fumiquinazoline C* em cultura de *A. fumigatus* em meio mínimo líquido por 72h, condição que apresenta menor esporulação (LIM; AMES; WALSH; KELLER, 2014). Neste estudo, podemos observar a ausência de *fumiquinazoline C* nas amostras de 72h em ML, mas a presença em amostras de 120h em ML, sugerindo um aumento da produção de maneira dependente do tempo. Além disso, sabe-se que metabólitos localizados em esporos podem contribuir para a virulência de fungos patogênicos (GAUTHIER; WANG; SIFUENTES DOS SANTOS; FYSIKOPOULOS *et*

al., 2012) e a presença desse metabólito em VEs pode estar relacionada a mudanças no ambiente em favor da germinação de esporos e sobrevivência fúngica.

Em relação à *fumitremorgin C*, relatos anteriores demonstram efeito de inibição da proliferação de células de câncer de próstata por desencadeamento de apoptose (KIM; KIM; PARK, 2017) e ressensibilização de células resistentes a múltiplas drogas usando modelos *in vitro* de câncer de mama e cólon (RABINDRAN; HE; SINGH; BROWN *et al.*, 1998; RABINDRAN; ROSS; DOYLE; YANG *et al.*, 2000). *Fumitremorgin C* também pode atenuar a formação e função dos osteoclastos e suprimir a atividade do fator nuclear NF- κ B, o nível de espécies reativas de oxigênio intracelular e a via da proteína quinase ativada por mitógeno (MAPK) (YUAN; CHEN; CHEN; WANG *et al.*, 2020). NF- κ B é ligado à via de sinalização pró-inflamatória, sendo ativado por citocinas pró-inflamatórias, levando à expressão de outros genes pró-inflamatórios e participando diretamente do recrutamento de leucócitos, sobrevivência celular e processo inflamatório (LAWRENCE, 2009). A ação inibitória de *fumitremorgin C* pode estar relacionada à prevenção da inflamação do hospedeiro por meio da supressão desta via, garantindo uma vantagem para a sobrevivência do fungo, conforme descrito para crocina (DU; CHI; SONG; DI *et al.*, 2018). Além disso, as cascatas das MAPK são importantes elementos de sinalização que regulam processos básicos, incluindo proliferação e diferenciação celular e respostas ao estresse, transmitindo sinais extracelulares para alvos intracelulares (GUO; PAN; LIU; SHEN *et al.*, 2020). Estudos *in vivo* sugeriram que a quinase regulada por sinal extracelular (ERK), membro da família MAPK, foi essencial na defesa contra a infecção por *A. fumigatus*, inibindo a síntese do fator de necrose tumoral alfa (TNF- α) e reduzindo a morte de conídios após estimulação (DUBOURDEAU; ATHMAN; BALLOY; HUERRE *et al.*, 2006). Essa atividade inibitória de *fumitremorgin C* também pode estar relacionada à garantia da germinação dos esporos e é uma hipótese concebível que esse componente natural tenha um papel importante na modulação das vias de sinalização celular do hospedeiro, que podem ser inibidas em um mecanismo de evasão durante a infecção.

Finalmente, *peroxyergosterol* é uma molécula conhecida por ter atividades amebicida (MEZA-MENCHACA; SUÁREZ-MEDELLÍN; DEL ÁNGEL-PIÑA; TRIGOS, 2015), antibacteriana (DUARTE; FERREIRA; MARTINS; VIVEIROS *et al.*, 2007; MERDIVAN; LINDEQUIST, 2017) e tripanocida (MEZA-MENCHACA; RAMOS-

LIGONIO; LÓPEZ-MONTEON; VIDAL LIMÓN *et al.*, 2019; MEZA-MENCHACA; SUÁREZ-MEDELLÍN; DEL ÁNGEL-PIÑA; TRIGOS, 2015; RAMOS-LIGONIO; LÓPEZ-MONTEON; TRIGOS, 2012), apresentando vasta ação biológica contra alguns organismos. Por exemplo, o tratamento com nível citotóxico de *peroxyergosterol* é relacionado com o aumento do tamanho de VEs produzidas por *Trypanosoma cruzi* e com o rompimento das membranas citoplasmática e nuclear e do aparelho de Golgi, devido a um mecanismo que pode envolver espécies reativas de oxigênio (MEZA-MENCHACA; RAMOS-LIGONIO; LÓPEZ-MONTEON; VIDAL LIMÓN *et al.*, 2019), sugerindo que a substituição de ergosterol por *peroxyergosterol* desencadeia uma série de reações de radicais livres na membrana do protozoário (MEZA-MENCHACA; RAMOS-LIGONIO; LÓPEZ-MONTEON; VIDAL LIMÓN *et al.*, 2019). A molécula também está relacionada à inibição do crescimento de células tumorais (EL-SHERIF; AHMED; IBRAHIM; HABIB *et al.*, 2020; MERDIVAN; LINDEQUIST, 2017; TAN; PAN; LIU; TIAN *et al.*, 2017; YANG; LUO; YASHENG; ZHAO *et al.*, 2020) por meio da interferência em diversas vias, como o aumento do fosforilação da proteína quinase PERK e do fator de iniciação eucariótico 2 (eIF2 α), e o aumento da expressão do fator de transcrição de ativação 4 (ATF4) (YANG; LUO; YASHENG; ZHAO *et al.*, 2020), tendo sua ação ligada a mecanismos que envolvem espécies reativas de oxigênio e estresse do retículo endoplasmático (YANG; LUO; YASHENG; ZHAO *et al.*, 2020). Ademais, é uma molécula conhecida por ser responsável por suprimir a resposta pró-inflamatória induzida pelo vírus *influenza A* em células epiteliais pulmonares humanas (A549), inibindo a ativação das vias de sinalização do gene I induzível por ácido retinóico citosólico (RIG-I), que envolvem p38 MAPK e NF- κ B, e prevenindo apoptose (ZHOU; LIANG; FENG; LI *et al.*, 2019). Anteriormente, foi relatado o envolvimento da expressão de MAPK e NF- κ B com aumento da carga fúngica e mortalidade (DUBOURDEAU; ATHMAN; BALLOY; HUERRE *et al.*, 2006; MEHRAD; STRIETER; STANDIFORD, 1999). Na verdade, *peroxyergosterol* atenuou a resposta pró-inflamatória do hospedeiro (ZHOU; LIANG; FENG; LI *et al.*, 2019), o que poderia permitir a sobrevivência do fungo nos pulmões. Além disso, foi sugerido que *peroxyergosterol* pode suprimir as respostas inflamatórias induzidas por lipopolissacarídeos de células semelhantes a macrófagos em camundongo através da inibição de NF- κ B e C/EBP β , uma proteína de ligação intensificadora de CCAAT, e, como consequência, suprimir a fosforilação de MAPKs

(KOBORI; YOSHIDA; OHNISHI-KAMEYAMA; SHINMOTO, 2007). Todas essas características, destacando prevenção da inflamação do hospedeiro e regulação das respostas ao estresse, levam a especular que a presença de *peroxyergosterol* nas VEs é outra estratégia para garantir a sobrevivência do fungo, principalmente por meio da modulação da resposta imune do hospedeiro e das vias de sinalização celular.

5.3. Importância da presença de RNA em VEs produzidas por *A. fumigatus*

A presença de RNA em VEs fúngicas foi relatada anteriormente, confirmando que as moléculas são carregadas para o espaço extracelular protegidas da hidrólise e da degradação por ação enzimática (PERES DA SILVA; PUCCIA; RODRIGUES; OLIVEIRA *et al.*, 2015), fato confirmado por esse estudo com relação à VEs produzidas por *A. fumigatus*. Estudos relatam que foram encontrados micro RNAs (miRNAs), RNAs não-codificantes (ncRNAs), RNAs mensageiros (mRNAs) e RNAs transportadores (tRNAs), relacionados à transporte mediado por vesículas, vias metabólicas importantes e outros processos biológicos, em diversas espécies de fungos como *Candida albicans*, *Cryptococcus* spp., *Paracoccidioides brasiliensis*, *Saccharomyces cerevisiae* e *Trichoderma reesei* (DE PAULA; ANTONIÊTO; NOGUEIRA; RIBEIRO *et al.*, 2019; JOFFE, LUNA S.; NIMRICHTER, LEONARDO; RODRIGUES, MARCIO L.; DEL POETA, MAURIZIO, 2016; PERES DA SILVA; PUCCIA; RODRIGUES; OLIVEIRA *et al.*, 2015; REIS; BORGES; JOZEFOWICZ; SENA *et al.*, 2019).

Se comparados a outros trabalhos que demonstram a presença de RNA em VEs (DE PAULA; ANTONIÊTO; NOGUEIRA; RIBEIRO *et al.*, 2019; REIS; BORGES; JOZEFOWICZ; SENA *et al.*, 2019), os perfis de amostras de RNA extraído das VEs produzidas por *A. fumigatus* diferem pouco, apresentando principalmente moléculas de RNA de tamanho menor. Neste caso, VEs produzidas por *A. fumigatus* carregam, em sua maioria, RNAs menores que 2000 nucleotídeos para o espaço extracelular, estando de acordo com a literatura.

Apesar de escassez nas informações sobre a função dessas moléculas nas VEs fúngicas, a presença de RNA em VEs é amplamente relatada para diversos organismos e é consistente com a hipótese levantada por outros estudos de que a exportação de ácidos nucleicos mediada por VEs interfere na expressão gênica em

células hospedeiras e fúngicas, sugerindo sua participação na comunicação entre células.

5.4. Características das proteínas encontradas em VEs produzidas por *A. fumigatus*

O perfil de proteínas contidas em VEs produzidas por *A. fumigatus* é pouco conhecido, mas estudos anteriores relataram 60 (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019) e 142 proteínas encontradas (RIZZO; CHAZE; MIRANDA; ROBERSON *et al.*, 2020). Nesse estudo, encontramos 218 proteínas presentes em VEs, que estão envolvidas nos mais diversos mecanismos fisiológicos do fungo. Foram encontradas enzimas e subunidades (catalases, desidrogenases, glucosidases, hidrolases, lipases, oxirredutases, peptidases, quinases, ATP sintases *etc.*), proteínas ribossomais, transportadoras de ADP/ATP e de parede celular, fatores de transcrição, histonas, componentes de proteassomos e diversas proteínas não caracterizadas.

Assim como reportado anteriormente, as atividades de hidrolase, de oxirredutase e estrutural são realizadas pela maior parte das proteínas obtidas em VEs analisadas, assim como existe uma quantidade razoável de proteínas com função desconhecida, catalítica e de regulação de processos biológicos (RIZZO; CHAZE; MIRANDA; ROBERSON *et al.*, 2020; SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019), conforme observado na Figura 9. Esse padrão também pode ser observado nas amostras de micélio obtidas neste estudo. Entretanto, a localização celular das proteínas encontradas em VEs tem uma alteração em relação ao estudo anterior, ao invés da maioria localizada na região extracelular e na parede celular (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019), as proteínas têm atuação localizada principalmente nos ribossomos e nas membranas, além de núcleo, citoplasma e mitocôndrias, também reportados anteriormente (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019), como podemos observar na Figura 10.

Figura 9. Atividades mais relevantes das proteínas identificadas.

Fonte: Produção do autor. O gráfico *lollipop* representa as 25 atividades com maior número de proteínas relacionadas. As proteínas foram identificadas em amostras de micélio (azul) e VEs (vermelho), e estão representadas em verde quando estão presentes nas duas amostras.

Figura 10. Localizações mais relevantes das proteínas identificadas.

Fonte: Produção do autor. O gráfico *lollipop* representa as localizações celulares relacionadas com as proteínas identificadas. Estão representadas amostras de micélio (azul), VEs (vermelho) e proteínas presentes simultaneamente nas duas amostras (verde).

Do total de proteínas obtidas, apenas 70 proteínas eram comuns às duas condições, micélio (38,88% do total) e VEs (32,11% do total). É possível ver na Figura 9 e na Figura 10 que a maioria das proteínas de cada função molecular e localização são independentes, sugerindo que as VEs carregam conteúdo diferenciado e podem ter atuação específica, separada dos processos desempenhados pelo micélio.

Em adição, as proteínas presentes nas amostras de micélio também pertenciam aos grupos de atividades identificadas para as VEs, além de apresentar

proteínas relacionadas com ciclo celular, parede celular, ligação de RNA e DNA, pigmentos conidiais e estresse térmico, importantes para sobrevivência e patogênese (ABAD; FERNÁNDEZ-MOLINA; BIKANDI; RAMÍREZ *et al.*, 2010). Ademais, as proteínas identificadas nessas amostras realizam atividades catalíticas, de ligação de ATP, GTP e íons metálicos e atividade antioxidante, estando localizadas principalmente em ribossomos, membrana, núcleo e citoplasma.

CONCLUSÕES

6. CONCLUSÕES

Este estudo busca seguir os primeiros passos para compreender a importância do transporte, mediado por VEs, dessas moléculas ao espaço extracelular e a relação com a comunicação, talvez até competição, do fungo com o ambiente e outros organismos. Dessa forma, começamos a ter meios para entender melhor a produção e composição destas VEs.

Após a etapa de padronização das técnicas de cultura, isolamento de VEs e extração do conteúdo interno das VEs, buscando conhecer melhor a composição das VEs de *A. fumigatus* e relacionar sua carga com funções fisiopatológicas do fungo, pudemos analisar os dados obtidos e propor que a presença de metabólitos secundários em VEs produzidas por *A. fumigatus* pode ser correlacionada com a atividade inibitória do fungo em diferentes vias do hospedeiro e a capacidade do fungo de lidar com condições ambientais adversas e favorecer a germinação de conídios, favorecendo a sobrevivência do fungo. Até o momento, este é o primeiro estudo demonstrando que VEs de *Aspergillus spp.* contêm metabólitos secundários e um dos primeiros demonstrando que *A. fumigatus* produz VEs em MS. Nossa hipótese é que essas moléculas transportadas pelas VEs podem estar envolvidas em processos importantes para a interação fungo-hospedeiro, bem como na interação com o meio ambiente. Ademais, a presença de RNA e proteínas nas VEs, além da diferença entre o seu conteúdo proteico e o das amostras de micélio, podem indicar um mecanismo fúngico especializado, que otimiza as chances de sobrevivência do fungo e potencializa a virulência num cenário de infecção.

Os resultados apresentados reforçam o conhecimento sobre a produção de VEs por *A. fumigatus* em meio líquido e sólido. Essas VEs podem transportar metabólitos secundários, RNAs e proteínas em seu interior. Além disso, os dados sugerem a importância do estudo das VEs fúngicas e que a sua produção e liberação para o meio externo podem estar correlacionadas à sobrevivência e patogenicidade do fungo, fortemente ligadas à modulação da resposta imune durante a infecção do hospedeiro.

REFERÊNCIAS⁵

⁵ De acordo com a Associação Brasileira de Normas Técnicas (ABNT 6023)

REFERÊNCIAS

- ABAD, A.; FERNÁNDEZ-MOLINA, J. V.; BIKANDI, J.; RAMÍREZ, A. *et al.* What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. **Rev Iberoam Micol**, 27, n. 4, p. 155-182, 2010 Oct-Dec 2010.
- ABELS, E. R.; BREAKFIELD, X. O. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. **Cell Mol Neurobiol**, 36, n. 3, p. 301-312, Apr 2016.
- ALBUQUERQUE, P. C.; NAKAYASU, E. S.; RODRIGUES, M. L.; FRASES, S. *et al.* Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. **Cell Microbiol**, 10, n. 8, p. 1695-1710, Aug 2008.
- ALMEIDA, F.; WOLF, J. M.; DA SILVA, T. A.; DELEON-RODRIGUEZ, C. M. *et al.* Galectin-3 impacts *Cryptococcus neoformans* infection through direct antifungal effects. **Nature Communications**, 8, DEC 6 2017 2017.
- ARASTEHFAR, A.; CARVALHO, A.; VAN DE VEERDONK, F. L.; JENKS, J. D. *et al.* COVID-19 Associated Pulmonary Aspergillosis (CAPA)-From Immunology to Treatment. **J Fungi (Basel)**, 6, n. 2, Jun 2020.
- BATAGOV, A. O.; KUROCHKIN, I. V. Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions. **Biol Direct**, 8, p. 12, Jun 2013.
- BELOFSKY, G. N.; ANGUERA, M.; JENSEN, P. R.; FENICAL, W. *et al.* Oxepinamides A-C and fumiquinazolines H--I: bioactive metabolites from a marine isolate of a fungus of the genus *Acremonium*. **Chemistry**, 6, n. 8, p. 1355-1360, Apr 2000.
- BENNETT, J. W. *Aspergillus*: a primer for the novice. **Med Mycol**, 47 Suppl 1, p. S5-12, 2009.
- BENNETT, J. W. An Overview of the Genus *Aspergillus*. *In*: MACHIDA, M. e GOMI, K. (Ed.). **Aspergillus: Molecular Biology and Genomics**: Caister Academic Press, 2010. cap. 1, p. 1-17.
- BENNETT, J. W.; KLICH, M. Mycotoxins. **Clin Microbiol Rev**, 16, n. 3, p. 497-516, Jul 2003.
- BIELSKA, E.; SISQUELLA, M. A.; ALDEIEG, M.; BIRCH, C. *et al.* Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen *Cryptococcus gattii*. **Nat Commun**, 9, n. 1, p. 1556, 04 2018.
- BINNS, D.; DIMMER, E.; HUNTLEY, R.; BARRELL, D. *et al.* QuickGO: a web-based tool for Gene Ontology searching. **Bioinformatics**, 25, n. 22, p. 3045-3046, 2009.

BITENCOURT, T. A.; REZENDE, C. P.; QUARESEMIN, N. R.; MORENO, P. *et al.* Extracellular Vesicles From the Dermatophyte. **Front Immunol**, 9, p. 2343, 2018.

BRAUER, V. S.; PESSONI, A. M.; BITENCOURT, T. A.; DE PAULA, R. G. *et al.* Extracellular Vesicles from *Aspergillus flavus* Induce M1 Polarization. **mSphere**, 5, n. 3, May 2020.

BROWN, L.; WOLF, J. M.; PRADOS-ROSALES, R.; CASADEVALL, A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. **Nat Rev Microbiol**, 13, n. 10, p. 620-630, Oct 2015.

CAI, Q.; QIAO, L.; WANG, M.; HE, B. *et al.* Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. **Science**, 360, n. 6393, p. 1126-1129, 06 2018.

CHAZALET, V.; DEBEAUPUIS, J. P.; SARFATI, J.; LORTHOLARY, J. *et al.* Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. **J Clin Microbiol**, 36, n. 6, p. 1494-1500, Jun 1998.

CHEN, Z.; YUAN, X.; BUCHANAN, P.; QUEK, S. Y. Isolation and determination of lipophilic mycochemicals from a New Zealand edible native mushroom *Herichium novae-zealandiae*. **Journal of Food Composition and Analysis**, 88, p. 103456, 2020.

CHOI, D. S.; KIM, D. K.; KIM, Y. K.; GHO, Y. S. Proteomics of extracellular vesicles: Exosomes and ectosomes. **Mass Spectrom Rev**, 34, n. 4, p. 474-490, 2015 Jul-Aug 2015.

COSTA, J. H.; WASSANO, C. I.; ANGOLINI, C. F. F.; SCHERLACH, K. *et al.* Antifungal potential of secondary metabolites involved in the interaction between citrus pathogens. **Sci Rep**, 9, n. 1, p. 18647, Dec 2019.

DA SILVA, T. A.; ROQUE-BARREIRA, M. C.; CASADEVALL, A.; ALMEIDA, F. Extracellular vesicles from *Paracoccidioides brasiliensis* induced M1 polarization in vitro. **Sci Rep**, 6, p. 35867, 10 2016.

DE PAULA, R. G.; ANTONIÊTO, A. C. C.; NOGUEIRA, K. M. V.; RIBEIRO, L. F. C. *et al.* Extracellular vesicles carry cellulases in the industrial fungus. **Biotechnol Biofuels**, 12, p. 146, 2019.

DU, J.; CHI, Y.; SONG, Z.; DI, Q. *et al.* Crocin reduces *Aspergillus fumigatus*-induced airway inflammation and NF- κ B signal activation. **J Cell Biochem**, 119, n. 2, p. 1746-1754, 02 2018.

DUARTE, N.; FERREIRA, M. J.; MARTINS, M.; VIVEIROS, M. *et al.* Antibacterial activity of ergosterol peroxide against *Mycobacterium tuberculosis*: dependence upon system and medium employed. **Phytother Res**, 21, n. 7, p. 601-604, Jul 2007.

DUBOURDEAU, M.; ATHMAN, R.; BALLOY, V.; HUERRE, M. *et al.* *Aspergillus fumigatus* induces innate immune responses in alveolar macrophages through the

MAPK pathway independently of TLR2 and TLR4. **J Immunol**, 177, n. 6, p. 3994-4001, Sep 2006.

EL-SHERIF, N. F.; AHMED, S. A.; IBRAHIM, A. K.; HABIB, E. S. *et al.* Ergosterol Peroxide from the Egyptian Red Lingzhi or Reishi Mushroom, *Ganoderma resinaceum* (Agaricomycetes), Showed Preferred Inhibition of MCF-7 over MDA-MB-231 Breast Cancer Cell Lines. **Int J Med Mushrooms**, 22, n. 4, p. 389-396, 2020.

FALLON, J. P.; REEVES, E. P.; KAVANAGH, K. The *Aspergillus fumigatus* toxin fumagillin suppresses the immune response of *Galleria mellonella* larvae by inhibiting the action of haemocytes. **Microbiology**, 157, n. Pt 5, p. 1481-1488, May 2011.

FORNAL, E.; PARFIENIUK, E.; CZECHKO, R.; BILINSKA-WIELGUS, N. *et al.* Fast and easy liquid chromatography–mass spectrometry method for evaluation of postharvest fruit safety by determination of mycotoxins: Fumitremorgin C and verruculogen. **Postharvest Biology and Technology**, 131, p. 46-54, 2017/09/01/ 2017.

FREIRE, P. **A importância do ato de ler: em três artigos que se completam**. São Paulo: Cortez, 2017. (Questões da nossa época. 978-85-249-2565-8.

FRISVAD, J. C.; RANK, C.; NIELSEN, K. F.; LARSEN, T. O. Metabolomics of *Aspergillus fumigatus*. **Med Mycol**, 47 Suppl 1, p. S53-71, 2009.

FULLER, K. K.; RHODES, J. C. Protein kinase A and fungal virulence: a sinister side to a conserved nutrient sensing pathway. **Virulence**, 3, n. 2, p. 109-121, 2012 Mar-Apr 2012.

GASTBOIS, A.; CLAVALD, C.; AIMANIANDA, V.; LATGÉ, J. P. *Aspergillus fumigatus*: cell wall polysaccharides, their biosynthesis and organization. **Future Microbiol**, 4, n. 5, p. 583-595, Jun 2009.

GAUTHIER, T.; WANG, X.; SIFUENTES DOS SANTOS, J.; FYSIKOPOULOS, A. *et al.* Trypacidin, a spore-borne toxin from *Aspergillus fumigatus*, is cytotoxic to lung cells. **PLoS One**, 7, n. 2, p. e29906, 2012.

GIACOMAZZI, J.; BAETHGEN, L.; CARNEIRO, L. C.; MILLINGTON, M. A. *et al.* The burden of serious human fungal infections in Brazil. **Mycoses**, 59, n. 3, p. 145-150, Mar 2016.

GOMEZ, P.; HACKETT, T. L.; MOORE, M. M.; KNIGHT, D. A. *et al.* Functional genomics of human bronchial epithelial cells directly interacting with conidia of *Aspergillus fumigatus*. **BMC Genomics**, 11, p. 358, Jun 2010.

GUO, Y. J.; PAN, W. W.; LIU, S. B.; SHEN, Z. F. *et al.* ERK/MAPK signalling pathway and tumorigenesis. **Exp Ther Med**, 19, n. 3, p. 1997-2007, Mar 2020.

GURUCEAGA, X.; PEREZ-CUESTA, U.; ABAD-DIAZ DE CERIO, A.; GONZALEZ, O. *et al.* Fumagillin, a Mycotoxin of. **Toxins (Basel)**, 12, n. 1, 12 2019.

HORIKOSHI, R.; GOTO, K.; MITOMI, M.; OYAMA, K. *et al.* Identification of pyripyropene A as a promising insecticidal compound in a microbial metabolite screening. **J Antibiot (Tokyo)**, 70, n. 3, p. 272-276, Mar 2017.

HORIKOSHI, R.; GOTO, K.; MITOMI, M.; OYAMA, K. *et al.* Insecticidal properties of pyripyropene A, a microbial secondary metabolite, against agricultural pests. **J Pestic Sci**, 43, n. 4, p. 266-271, Nov 2018.

HUNTZINGER, E.; IZAURRALDE, E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. **Nat Rev Genet**, 12, n. 2, p. 99-110, Feb 2011.

IGUCHI, H.; KOSAKA, N.; OCHIYA, T. Secretory microRNAs as a versatile communication tool. **Commun Integr Biol**, 3, n. 5, p. 478-481, Sep 2010.

IKEDA, M. A. K.; DE ALMEIDA, J. R. F.; JANNUZZI, G. P.; CRONEMBERGER-ANDRADE, A. *et al.* Extracellular Vesicles From. **Front Microbiol**, 9, p. 2286, 2018.

JOFFE, L. S.; NIMRICHTER, L.; RODRIGUES, M. L.; DEL POETA, M. Potential Roles of Fungal Extracellular Vesicles during Infection. **mSphere**, 1, n. 4, 2016 Jul-Aug 2016.

JOFFE, L. S.; NIMRICHTER, L.; RODRIGUES, M. L.; DEL POETA, M. Potential Roles of Fungal Extracellular Vesicles during Infection. **mSphere**, 1, n. 4, p. e00099-00016, 2016.

KAMEI, K.; WATANABE, A. Aspergillus mycotoxins and their effect on the host. **Med Mycol**, 43 Suppl 1, p. S95-99, May 2005.

KANAANI, H.; HARGREAVES, M.; RISTOVSKI, Z.; MORAWSKA, L. Deposition rates of fungal spores in indoor environments, factors effecting them and comparison with non-biological aerosols. **Atmospheric Environment**, 42, n. 30, p. 7141-7154, 2008.

KIM, K. M.; ABDELMOHSEN, K.; MUSTAPIC, M.; KAPOGIANNIS, D. *et al.* RNA in extracellular vesicles. **Wiley Interdiscip Rev RNA**, 8, n. 4, 07 2017.

KIM, Y. S.; KIM, S. K.; PARK, S. J. Apoptotic effect of demethoxyfumitremorgin C from marine fungus Aspergillus fumigatus on PC3 human prostate cancer cells. **Chem Biol Interact**, 269, p. 18-24, May 2017.

KNIEMEYER, O.; LESSING, F.; BRAKHAGE, A. A. Proteome analysis for pathogenicity and new diagnostic markers for Aspergillus fumigatus. **Med Mycol**, 47 Suppl 1, p. S248-254, 2009.

KOBORI, M.; YOSHIDA, M.; OHNISHI-KAMEYAMA, M.; SHINMOTO, H. Ergosterol peroxide from an edible mushroom suppresses inflammatory responses in RAW264.7 macrophages and growth of HT29 colon adenocarcinoma cells. **Br J Pharmacol**, 150, n. 2, p. 209-219, Jan 2007.

KUBO, H. Extracellular Vesicles in Lung Disease. **Chest**, 153, n. 1, p. 210-216, 01 2018.

KUIPERS, M. E.; HOKKE, C. H.; SMITS, H. H.; NOLTE-'T HOEN, E. N. M. Pathogen-Derived Extracellular Vesicle-Associated Molecules That Affect the Host Immune System: An Overview. **Front Microbiol**, 9, p. 2182, 2018.

KWON-CHUNG, K. J.; SUGUI, J. A. What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*? **Med Mycol**, 47 Suppl 1, p. S97-103, 2009.

KWON-CHUNG, K. J.; SUGUI, J. A. *Aspergillus fumigatus*--what makes the species a ubiquitous human fungal pathogen? **PLoS Pathog**, 9, n. 12, p. e1003743, 2013.

LATGÉ, J. P. *Aspergillus fumigatus* and aspergillosis. **Clin Microbiol Rev**, 12, n. 2, p. 310-350, Apr 1999.

LATGÉ, J. P. The pathobiology of *Aspergillus fumigatus*. **Trends Microbiol**, 9, n. 8, p. 382-389, Aug 2001.

LAWRENCE, T. The nuclear factor NF-kappaB pathway in inflammation. **Cold Spring Harb Perspect Biol**, 1, n. 6, p. a001651, Dec 2009.

LEE, K. R.; CHAE, S. H.; KIM, M. J.; CHAE, Y. J. *et al.* Determination of *Penicillium griseofulvum*-oriented pyripyropene A, a selective inhibitor of acyl-coenzyme A:cholesterol acyltransferase 2, in mouse plasma using liquid chromatography-tandem mass spectrometry and its application to pharmacokinetic studies. **Biomed Chromatogr**, 33, n. 2, p. e4388, Feb 2019.

LEICHTER, C. A.; THOMPSON, N.; JOHNSON, B. R.; SCOTT, J. G. The high potency of ME-5343 to aphids is due to a unique mechanism of action. *Pesticide Biochemistry and Physiology: Elsevier*. 107: 169-176 p. 2013.

LEIPHEIMER, J.; BLOOM, A. L. M.; PANEPINTO, J. C. Protein Kinases at the Intersection of Translation and Virulence. **Front Cell Infect Microbiol**, 9, p. 318, 2019.

LIM, F. Y.; AMES, B.; WALSH, C. T.; KELLER, N. P. Co-ordination between BrIA regulation and secretion of the oxidoreductase FmqD directs selective accumulation of fumiquinazoline C to conidial tissues in *Aspergillus fumigatus*. **Cell Microbiol**, 16, n. 8, p. 1267-1283, Aug 2014.

LONG, S.; RESENDE, D. I. S. P.; KIJJOA, A.; SILVA, A. M. S. *et al.* Synthesis of New Proteomimetic Quinazolinone Alkaloids and Evaluation of Their Neuroprotective and Antitumor Effects. **Molecules**, 24, n. 3, Feb 2019.

MAHIEU, L. M.; DE DOOY, J. J.; VAN LAER, F. A.; JANSSENS, H. *et al.* A prospective study on factors influencing aspergillus spore load in the air during renovation works in a neonatal intensive care unit. **J Hosp Infect**, 45, n. 3, p. 191-197, Jul 2000.

MATSUDA, D.; OHSHIRO, T.; OHTAWA, M.; YAMAZAKI, H. *et al.* In vitro metabolism of pyripyropene A and ACAT inhibitory activity of its metabolites. **J Antibiot (Tokyo)**, 68, n. 1, p. 27-34, Jan 2015.

MEHRAD, B.; STRIETER, R. M.; STANDIFORD, T. J. Role of TNF- α in Pulmonary Host Defense in Murine Invasive Aspergillosis. **The Journal of Immunology**, 162, n. 3, p. 1633-1640, 1999.

MERDIVAN, S.; LINDEQUIST, U. Ergosterol Peroxide: A Mushroom-Derived Compound with Promising Biological Activities-A Review. **Int J Med Mushrooms**, 19, n. 2, p. 93-105, 2017.

MEZA-MENCHACA, T.; RAMOS-LIGONIO, A.; LÓPEZ-MONTEON, A.; VIDAL LIMÓN, A. *et al.* Insights into Ergosterol Peroxide's Trypanocidal Activity. **Biomolecules**, 9, n. 9, 09 2019.

MEZA-MENCHACA, T.; SUÁREZ-MEDELLÍN, J.; DEL ÁNGEL-PIÑA, C.; TRIGOS, Á. The Amoebicidal Effect of Ergosterol Peroxide Isolated from *Pleurotus ostreatus*. **Phytother Res**, 29, n. 12, p. 1982-1986, Dec 2015.

MOURA, S.; CERQUEIRA, L.; ALMEIDA, A. Invasive pulmonary aspergillosis: current diagnostic methodologies and a new molecular approach. **Eur J Clin Microbiol Infect Dis**, 37, n. 8, p. 1393-1403, Aug 2018.

NASIR, N.; FAROOQI, J.; MAHMOOD, S. F.; JABEEN, K. COVID-19-associated pulmonary aspergillosis (CAPA) in patients admitted with severe COVID-19 pneumonia: An observational study from Pakistan. **Mycoses**, 63, n. 8, p. 766-770, Aug 2020.

NIELSON, J. R.; FREDRICKSON, E. K.; WALLER, T. C.; RENDÓN, O. Z. *et al.* Sterol Oxidation Mediates Stress-Responsive Vms1 Translocation to Mitochondria. **Mol Cell**, 68, n. 4, p. 673-685.e676, Nov 2017.

OHSHIRO, T.; MATSUDA, D.; SAKAI, K.; DEGIROLAMO, C. *et al.* Pyripyropene A, an acyl-coenzyme A:cholesterol acyltransferase 2-selective inhibitor, attenuates hypercholesterolemia and atherosclerosis in murine models of hyperlipidemia. **Arterioscler Thromb Vasc Biol**, 31, n. 5, p. 1108-1115, May 2011.

PAYNE, G. A.; BROWN, M. P. Genetics and physiology of aflatoxin biosynthesis. **Annu Rev Phytopathol**, 36, p. 329-362, 1998.

PERES DA SILVA, R.; PUCCIA, R.; RODRIGUES, M. L.; OLIVEIRA, D. L. *et al.* Extracellular vesicle-mediated export of fungal RNA. **Sci Rep**, 5, p. 7763, Jan 2015.

PERKINS, D. N.; PAPPIN, D. J.; CREASY, D. M.; COTTRELL, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. **Electrophoresis**, 20, n. 18, p. 3551-3567, Dec 1999.

PERRONE, G.; GALLO, A. *Aspergillus* Species and Their Associated Mycotoxins. **Methods Mol Biol**, 1542, p. 33-49, 2017.

PINI, G.; FAGGI, E.; DONATO, R.; SACCO, C. *et al.* Invasive pulmonary aspergillosis in neutropenic patients and the influence of hospital renovation. **Mycoses**, 51, n. 2, p. 117-122, Mar 2008.

RABINDRAN, S. K.; HE, H.; SINGH, M.; BROWN, E. *et al.* Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C. **Cancer Res**, 58, n. 24, p. 5850-5858, Dec 1998.

RABINDRAN, S. K.; ROSS, D. D.; DOYLE, L. A.; YANG, W. *et al.* Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. **Cancer Res**, 60, n. 1, p. 47-50, Jan 2000.

RAMOS-LIGONIO, A.; LÓPEZ-MONTEON, A.; TRIGOS, A. Trypanocidal activity of ergosterol peroxide from *Pleurotus ostreatus*. **Phytother Res**, 26, n. 6, p. 938-943, Jun 2012.

RAPPSILBER, J.; MANN, M.; ISHIHAMA, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. **Nat Protoc**, 2, n. 8, p. 1896-1906, 2007.

REALES-CALDERÓN, J. A.; VAZ, C.; MONTEOLIVA, L.; MOLERO, G. *et al.* *Candida albicans* Modifies the Protein Composition and Size Distribution of THP-1 Macrophage-Derived Extracellular Vesicles. **J Proteome Res**, 16, n. 1, p. 87-105, 01 2017.

REIJULA, K.; TUOMI, T. Mycotoxins of aspergilli: exposure and health effects. **Front Biosci**, 8, p. s232-235, May 2003.

REIS, F. C. G.; BORGES, B. S.; JOZEFOWICZ, L. J.; SENA, B. A. G. *et al.* A Novel Protocol for the Isolation of Fungal Extracellular Vesicles Reveals the Participation of a Putative Scramblase in Polysaccharide Export and Capsule Construction in. **mSphere**, 4, n. 2, 03 2019.

RESENDE, D. I. S. P.; BOONPOTHONG, P.; SOUSA, E.; KIJJOA, A. *et al.* Chemistry of the fumiquinazolines and structurally related alkaloids. **Nat Prod Rep**, 36, n. 1, p. 7-34, 01 2019.

RIZZO, J.; CHAZE, T.; MIRANDA, K.; ROBERSON, R. W. *et al.* Characterization of Extracellular Vesicles Produced by *Aspergillus fumigatus* Protoplasts. **mSphere**, 5, n. 4, p. e00476-00420, 2020.

RODRIGUES, M. L.; CASADEVALL, A. A two-way road: novel roles for fungal extracellular vesicles. **Mol Microbiol**, 110, n. 1, p. 11-15, 10 2018.

RODRIGUES, M. L.; GODINHO, R. M.; ZAMITH-MIRANDA, D.; NIMRICHTER, L. Traveling into Outer Space: Unanswered Questions about Fungal Extracellular Vesicles. **PLoS Pathog**, 11, n. 12, p. e1005240, Dec 2015.

RODRIGUES, M. L.; NAKAYASU, E. S.; ALMEIDA, I. C.; NIMRICHTER, L. The impact of proteomics on the understanding of functions and biogenesis of fungal extracellular vesicles. **J Proteomics**, 97, p. 177-186, Jan 2014.

RODRIGUES, M. L.; NAKAYASU, E. S.; OLIVEIRA, D. L.; NIMRICHTER, L. *et al.* Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. **Eukaryot Cell**, 7, n. 1, p. 58-67, Jan 2008.

RODRIGUES, M. L.; NIMRICHTER, L.; OLIVEIRA, D. L.; FRASES, S. *et al.* Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. **Eukaryot Cell**, 6, n. 1, p. 48-59, Jan 2007.

RUDEL, L. L.; LEE, R. G.; PARINI, P. ACAT2 is a target for treatment of coronary heart disease associated with hypercholesterolemia. **Arterioscler Thromb Vasc Biol**, 25, n. 6, p. 1112-1118, Jun 2005.

SILVA, M. G.; FURTADO, N. A.; PUPO, M. T.; FONSECA, M. J. *et al.* Antibacterial activity from *Penicillium corylophilum* Dierckx. **Microbiol Res**, 159, n. 4, p. 317-322, 2004.

SOTO, G.; STRITZLER, M.; LISI, C.; ALLEVA, K. *et al.* Acetoacetyl-CoA thiolase regulates the mevalonate pathway during abiotic stress adaptation. **J Exp Bot**, 62, n. 15, p. 5699-5711, Nov 2011.

SOUZA, J. A. M.; BALTAZAR, L. M.; CARREGAL, V. M.; GOUVEIA-EUFRASIO, L. *et al.* Characterization of. **Front Microbiol**, 10, p. 2008, 2019.

SUBEDI, P.; SCHNEIDER, M.; PHILIPP, J.; AZIMZADEH, O. *et al.* Comparison of methods to isolate proteins from extracellular vesicles for mass spectrometry-based proteomic analyses. **Anal Biochem**, 584, p. 113390, Nov 2019.

SUGUI, J. A.; KIM, H. S.; ZAREMBER, K. A.; CHANG, Y. C. *et al.* Genes differentially expressed in conidia and hyphae of *Aspergillus fumigatus* upon exposure to human neutrophils. **PLoS One**, 3, n. 7, p. e2655, Jul 2008.

SUGUI, J. A.; KWON-CHUNG, K. J.; JUVVADI, P. R.; LATGÉ, J. P. *et al.* *Aspergillus fumigatus* and related species. **Cold Spring Harb Perspect Med**, 5, n. 2, p. a019786, Nov 2014.

SUGUI, J. A.; PARDO, J.; CHANG, Y. C.; ZAREMBER, K. A. *et al.* Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. **Eukaryot Cell**, 6, n. 9, p. 1562-1569, Sep 2007.

SZALEWSKI, D. A.; HINRICHS, V. S.; ZINNIEL, D. K.; BARLETTA, R. G. The pathogenicity of *Aspergillus fumigatus*, drug resistance, and nanoparticle delivery. **Can J Microbiol**, 64, n. 7, p. 439-453, Jul 2018.

- TAKAHASHI, C.; MATSUSHITA, T.; DOI, M.; MINOURA, K. *et al.* Fumiquinazolines A–G, novel metabolites of a fungus separated from a *Pseudolabrus* marine fish. *Journal of the Chemical Society, Perkin Transactions 1* 1995.
- TAN, W.; PAN, M.; LIU, H.; TIAN, H. *et al.* Ergosterol peroxide inhibits ovarian cancer cell growth through multiple pathways. **Onco Targets Ther**, 10, p. 3467-3474, 2017.
- THÉRY, C.; WITWER, K. W.; AIKAWA, E.; ALCARAZ, M. J. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. **J Extracell Vesicles**, 7, n. 1, p. 1535750, 2018.
- TKACH, M.; THÉRY, C. Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. **Cell**, 164, n. 6, p. 1226-1232, Mar 2016.
- TOMODA, H.; TABATA, N.; YANG, D. J.; NAMATAME, I. *et al.* Pyripyropenes, novel ACAT inhibitors produced by *Aspergillus fumigatus*. IV. Structure elucidation of pyripyropenes M to R. **J Antibiot (Tokyo)**, 49, n. 3, p. 292-298, Mar 1996.
- TRICARICO, C.; CLANCY, J.; D'SOUZA-SCHOREY, C. Biology and biogenesis of shed microvesicles. **Small GTPases**, 8, n. 4, p. 220-232, 10 2017.
- VALLEJO, M. C.; MATSUO, A. L.; GANIKO, L.; MEDEIROS, L. C. *et al.* The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic α -Galactosyl epitopes. **Eukaryot Cell**, 10, n. 3, p. 343-351, Mar 2011.
- VAN NIEL, G.; D'ANGELO, G.; RAPOSO, G. Shedding light on the cell biology of extracellular vesicles. **Nat Rev Mol Cell Biol**, 19, n. 4, p. 213-228, 04 2018.
- VARGAS, G.; ROCHA, J. D.; OLIVEIRA, D. L.; ALBUQUERQUE, P. C. *et al.* Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. **Cell Microbiol**, 17, n. 3, p. 389-407, Mar 2015.
- VOLTERSEN, V.; BLANGO, M. G.; HERRMANN, S.; SCHMIDT, F. *et al.* Proteome Analysis Reveals the Conidial Surface Protein CcpA Essential for Virulence of the Pathogenic Fungus. **mBio**, 9, n. 5, 10 2018.
- WANG, M.; CARVER, J. J.; PHELAN, V. V.; SANCHEZ, L. M. *et al.* Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. **Nat Biotechnol**, 34, n. 8, p. 828-837, 08 2016.
- WICKHAM, H. **ggplot2: Elegant Graphics for Data Analysis**. Springer-Verlag New York, 2016. 978-3-319-24277-4.
- WISEMAN, A. C. Immunosuppressive Medications. **Clin J Am Soc Nephrol**, 11, n. 2, p. 332-343, Feb 2016.
- YANG, Y.; LUO, X.; YASHENG, M.; ZHAO, J. *et al.* Ergosterol peroxide from *Pleurotus ferulae* inhibits gastrointestinal tumor cell growth through induction of

apoptosis via reactive oxygen species and endoplasmic reticulum stress. **Food Funct**, 11, n. 5, p. 4171-4184, May 2020.

YUAN, Y.; CHEN, K.; CHEN, X.; WANG, C. *et al.* Fumitremorgin C Attenuates Osteoclast Formation and Function. **Front Pharmacol**, 11, p. 238, 2020.

ZARNOWSKI, R.; SANCHEZ, H.; COVELLI, A. S.; DOMINGUEZ, E. *et al.* Candida albicans biofilm-induced vesicles confer drug resistance through matrix biogenesis. **PLoS Biol**, 16, n. 10, p. e2006872, 10 2018.

ZHANG, Y.; WEI, W.; FAN, J.; JIN, C. *et al.* Aspergillus fumigatus Mitochondrial Acetyl Coenzyme A Acetyltransferase as an Antifungal Target. **Appl Environ Microbiol**, 86, n. 7, 03 2020.

ZHOU, B.; LIANG, X.; FENG, Q.; LI, J. *et al.* Ergosterol peroxide suppresses influenza A virus-induced pro-inflammatory response and apoptosis by blocking RIG-I signaling. **Eur J Pharmacol**, 860, p. 172543, Oct 2019.

APÊNDICE A

Imagens: Cromatogramas de íons extraídos do extrato de VEs de *A. fumigatus*.

Figura S1. Cromatogramas de íons extraídos de m/z 584,24 para (A) extrato de VEs de *A. fumigatus* e (B) análise de controle de MS. (C) Espectro de massa do íon $[M + H]^+$ m/z 584,2492 obtido para o PPPA (erro = -0,5 ppm) a 8,4 min. (D) Espectro MS/MS de íon $[M + H]^+$ m/z 584,2490. Fonte: Produção do autor.

Figura S2. Cromatogramas de íons extraídos de m/z 540,25 para (A) extrato de VEs de *A. fumigatus* e (B) análise de controle de MS. (C) Espectro de massa do íon $[M + H]^+$ m/z 540,2598 obtido para o PPPQ (erro = 0,2 ppm) a 8,6 min. Fonte: Produção do autor.

Figura S3. Cromatogramas de íons extraídos de m/z 380,19 para (A) extrato de VEs de *A. fumigatus* e (B) análise de controle de MS. (C) Espectro de massa de íon $[M + H]^+$ m/z 380,1970 obtido para *fumitremorgin C* (erro = -1,1 ppm) em 7,8 min. (D) Espectro MS / MS de íon $[M + H]^+$ m/z 380,1970. Fonte: Produção do autor.

Figura S4. Cromatogramas de íons extraídos de m/z 542,23 para (A) extrato de VEs de *A. fumigatus* e (B) análise de controle de MS. (C) Espectro de massa de íon $[M + H]^+$ m/z 542,2388 obtido para DPPPA (erro = -0,4 ppm) em 5,4 min. (D) Espectro MS/MS de íon $[M + H]^+$ m/z 542,2386. Fonte: Produção do autor.

Figura S5. Cromatogramas de íons extraídos de m/z 426,15 para (A) extrato de VEs de *A. fumigatus* e (B) análise de controle de MS. (C) Espectro de massa de íon $[M + H]^+$ m/z 426,1558 obtido para *fumiquinazoline C* (erro = -1,9 ppm) em 5,9 min. (D) Espectro MS/MS de íon $[M + H]^+$ m/z 426,1561. Fonte: Produção do autor.

Figura S6. Cromatogramas de íons extraídos de m/z 429,33 para (A) extrato de VEs de *A. fumigatus* e (B) análise de controle de MS. (C) Espectro de massa de íon $[M + H]^+$ m/z 429,3364 obtido para *peroxyergosterol* (erro = -0,9 ppm) em 10,0 min. (D) Espectro MS/MS de íon $[M + H]^+$ m/z 429,3366. Fonte: Produção do autor.

APÊNDICE B

Imagens: Correspondência entre banco de dados GNPS e o extrato de VEs.

Figura S7. Correspondência de MS/MS para PPPA entre o banco de dados GNPS (verde) e o extrato de VEs de *A. fumigatus* (preto). Fonte: Produção do autor.

Figura S8. Correspondência de MS/MS para DPPP A entre o banco de dados GNPS (verde) e o extrato de VEs de *A. fumigatus* (preto). Fonte: Produção do autor.

Figura S9. Correspondência de MS/MS para *fumiquinazoline C* entre o banco de dados GNPS (verde) e o extrato de VEs de *A. fumigatus* (preto). Fonte: Produção do autor.

Figura S10. Correspondência de MS/MS para *peroxyergosterol* entre o banco de dados GNPS (verde) e o extrato de VEs de *A. fumigatus* (preto). Fonte: Produção do autor.

APÊNDICE C

Tabelas: Listas combinadas de proteínas identificadas após espectrometria de massas.

Tabela S1. Lista combinada de proteínas identificadas em amostras de micélio e de VEs produzidas por *A. fumigatus*. ID e descrição foram obtidos pelo mecanismo de busca Mascot.

Proteína	ID	Descrição
P1	A0A0J5PGE8	Alpha-glucosidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_09020 PE=3 SV=1
P2	A0A0J5PHC9	C2 domain-containing protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08402 PE=4 SV=1
P3	A0A0J5PHJ6	Aldehyde dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08802 PE=3 SV=1
P4	A0A0J5PIC6	40S ribosomal protein S13 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08554 PE=3 SV=1
P5	A0A0J5PJR5	ATP synthase subunit alpha OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05553 PE=3 SV=1
P6	A0A0J5PKY1	60S acidic ribosomal protein P0 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06787 PE=3 SV=1
P7	A0A0J5PL15	Alcohol dehydrogenase, zinc-containing OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06827 PE=3 SV=1
P8	A0A0J5PL23	60S ribosomal protein L20 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06837 PE=3 SV=1
P9	A0A0J5PLK7	Proteasome subunit alpha type OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08610 PE=3 SV=1
P10	A0A0J5PMH5	Hsp70 chaperone BiP/Kar2 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03241 PE=3 SV=1
P11	A0A0J5PNA9	Ribosomal protein L16a OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06701 PE=3 SV=1
P12	A0A0J5PNR7	60S ribosomal protein L7 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03503 PE=4 SV=1
P13	A0A0J5PP11	60S ribosomal protein L12 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06936 PE=3 SV=1
P14	A0A0J5PPB2	Cell wall protein PhiA OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07778 PE=4 SV=1
P15	A0A0J5PPI2	Catalase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07858 PE=3 SV=1
P16	A0A0J5PPP6	ADP,ATP carrier protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06760 PE=3 SV=1
P17	A0A0J5PQZ1	Nucleoside diphosphate kinase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00307 PE=3 SV=1
P18	A0A0J5PRI0	Vacuolar aspartyl aminopeptidase Lap4 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00354 PE=3 SV=1
P19	A0A0J5PRP3	60S ribosomal protein L23 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00419 PE=3 SV=1
P20	A0A0J5PUR5	60S ribosomal protein L4 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00265 PE=4 SV=1

P21	A0A0J5PUT7	Ubiquitin C-terminal hydrolase (HAUSP) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00285 PE=3 SV=1
P22	A0A0J5PV41	Citrate synthase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00380 PE=3 SV=1
P23	A0A0J5PVC2	60S ribosomal protein L21 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01852 PE=4 SV=1
P24	A0A0J5PVL4	ATP synthase subunit beta OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00886 PE=3 SV=1
P25	A0A0J5PWT3	60S ribosomal protein L22, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01326 PE=4 SV=1
P26	A0A0J5PXT7	MFS transporter OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01270 PE=4 SV=1
P27	A0A0J5PXU7	60S ribosomal protein L32 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02239 PE=4 SV=1
P28	A0A0J5PYS3	Histone H2B OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02001 PE=3 SV=1
P29	A0A0J5PYY4	60S ribosomal protein L30 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02897 PE=4 SV=1
P30	A0A0J5PZ62	Aspartyl aminopeptidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01705 PE=3 SV=1
P31	A0A0J5PZF5	60S ribosomal protein L18 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03042 PE=4 SV=1
P32	A0A0J5Q2V6	V-type ATPase, B subunit OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02550 PE=3 SV=1
P33	A0A0J5Q3I8	Aldehyde dehydrogenase AldA OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04278 PE=3 SV=1
P34	A0A0J5Q3P8	FAD binding domain-containing protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04338 PE=3 SV=1
P35	A0A0J5Q3R6	NADH-ubiquinone oxidoreductase subunit OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04358 PE=4 SV=1
P36	A0A0J5Q3X4	60S ribosomal protein L7A OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04423 PE=4 SV=1
P37	A0A0J5Q435	Histone H4 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04988 PE=3 SV=1
P38	A0A0J5Q4A2	G-protein complex alpha subunit GpaA/FadA OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05053 PE=4 SV=1
P39	A0A0J5Q4C3	60S ribosomal protein I5 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05078 PE=3 SV=1
P40	A0A0J5Q552	Histone H3 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04987 PE=3 SV=1
P41	A0A0J5Q747	60S ribosomal protein L31e OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04445 PE=4 SV=1
P42	A0A0J5Q863	Carboxypeptidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04474 PE=3 SV=1
P43	A0A0J5SCL3	60S ribosomal protein L11 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06161 PE=3 SV=1
P44	A0A0J5SFG0	40S ribosomal protein S22 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_09056 PE=3 SV=1
P45	A0A0J5SFV2	Chromosome Unknown scaffold_14, whole genome shotgun sequence OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08801 PE=4 SV=1
P46	A0A0J5SG85	Lipase, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08423 PE=4 SV=1

P47	A0A0J5SHU1	ATPase subunit ATP4 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05542 PE=4 SV=1
P48	A0A0J5SJ64	Beta-glucosidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06723 PE=3 SV=1
P49	A0A0J5SJ77	40S ribosomal protein S3 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06738 PE=3 SV=1
P50	A0A0J5SJL9	Glutamate carboxypeptidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06903 PE=4 SV=1
P51	A0A0J5SLJ0	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03597 PE=4 SV=1
P52	A0A0J5SP09	40S ribosomal protein S1 OS=Aspergillus fumigatus Z5 OX=1437362 GN=RPS1 PE=3 SV=1
P53	A0A0J5SUQ0	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02323 PE=4 SV=1
P54	A0A0J5SWA6	Dipeptidyl-peptidase 5 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02913 PE=4 SV=1
P55	A0A0J5SXN1	Processing peptidase beta subunit OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04945 PE=3 SV=1
P56	A0A229X7A8	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_08900 PE=3 SV=1
P57	A0A229Y3W9	60S ribosomal protein L13 OS=Neosartorya fumigata OX=746128 GN=CDV57_03346 PE=3 SV=1
P58	A0A229Y455	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_03203 PE=3 SV=1
P59	A0A229Y7C2	Alpha-mannosidase OS=Neosartorya fumigata OX=746128 GN=CDV57_03634 PE=3 SV=1
P60	A1C4U0	Vacuolar aspartyl aminopeptidase Lap4, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_001190 PE=3 SV=1
P61	A1CMF7	Ribosomal protein L14 OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_096780 PE=4 SV=1
P62	A1D0L3	Oxidoreductase, short-chain dehydrogenase/reductase family, putative OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_041110 PE=4 SV=1
P63	A1DG36	Protein disulfide-isomerase OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_082940 PE=3 SV=1
P64	A1DGJ8	Secreted dipeptidyl peptidase OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_084570 PE=4 SV=1
P65	B0XPJ7	60S ribosomal protein L27e OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_006720 PE=4 SV=1
P66	B0XPK2	Elongation factor 1-alpha OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_006770 PE=3 SV=1
P67	B0XQ32	60S ribosomal protein L9, putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_008520 PE=4 SV=1

P68	B0XYU2	40S ribosomal protein S9 OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_042090 PE=4 SV=1
P69	Q5BEL6	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN1014.2 PE=4 SV=1
P70	Q5BFJ2	Transketolase OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN0688.2 PE=3 SV=1

Fonte: Produção do autor.

Tabela S2. Lista combinada de proteínas identificadas em réplicas de micélio de *A. fumigatus*. ID e descrição foram obtidos pelo mecanismo de busca Mascot.

Proteína	ID	Descrição
M1	A0A0J5PDI6	60S ribosomal protein L36 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06132 PE=3 SV=1
M2	A0A0J5PDS6	Antioxidant protein LsfA OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06242 PE=4 SV=1
M3	A0A0J5PE90	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06315 PE=4 SV=1
M4	A0A0J5PFJ6	Cobalamin-independent methionine synthase MetH/D OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06124 PE=3 SV=1
M5	A0A0J5PFS8	Catalase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07313 PE=3 SV=1
M6	A0A0J5PFY6	Allergen Asp F3 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07471 PE=4 SV=1
M7	A0A0J5PG48	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07398 PE=4 SV=1
M8	A0A0J5PHF0	Actin OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07225 PE=3 SV=1
M9	A0A0J5PHQ6	ATP synthase subunit d, mitochondrial OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07320 PE=3 SV=1
M10	A0A0J5PHR2	UTP--glucose-1-phosphate uridylyltransferase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08882 PE=3 SV=1
M11	A0A0J5PIX3	Nascent polypeptide-associated complex (NAC) subunit OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07319 PE=4 SV=1
M12	A0A0J5PJ44	BAP31 domain-containing protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_09144 PE=4 SV=1
M13	A0A0J5PKR9	40S ribosomal protein S12 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06752 PE=3 SV=1
M14	A0A0J5PKV1	Putative membrane bound C2 domain-containing protein (Vp115) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08883 PE=4 SV=1
M15	A0A0J5PL40	40S ribosomal protein S8 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06857 PE=3 SV=1
M16	A0A0J5PLK0	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07037 PE=4 SV=1
M17	A0A0J5PLU5	ATP synthase subunit gamma OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06924 PE=3 SV=1

M18	A0A0J5PLY5	6-phosphogluconolactonase, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06974 PE=4 SV=1
M19	A0A0J5PMW0	Fumarate reductase Osm1 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05534 PE=4 SV=1
M20	A0A0J5PN72	Elongation factor 1-alpha OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06666 PE=3 SV=1
M21	A0A0J5PNF3	NAD-specific glutamate dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03373 PE=3 SV=1
M22	A0A0J5PP10	Methyltransferase SirN-like protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05919 PE=4 SV=1
M23	A0A0J5PQ18	Inosine-5'-monophosphate dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03145 PE=3 SV=1
M24	A0A0J5PQ68	Allergen Asp F4 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03165 PE=4 SV=1
M25	A0A0J5PQV7	Riboflavin-specific deaminase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03415 PE=4 SV=1
M26	A0A0J5PQZ5	Thioredoxin reductase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00312 PE=4 SV=1
M27	A0A0J5PR44	Antigen OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00199 PE=4 SV=1
M28	A0A0J5PRK0	Molecular chaperone OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00374 PE=3 SV=1
M29	A0A0J5PRR1	Protein disulfide-isomerase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03389 PE=3 SV=1
M30	A0A0J5PRZ6	HLH DNA binding protein (Penr2), putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00647 PE=4 SV=1
M31	A0A0J5PSK0	Superoxide dismutase [Cu-Zn] OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00764 PE=3 SV=1
M32	A0A0J5PSR3	Cytochrome b-c1 complex subunit Rieske, mitochondrial OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00892 PE=4 SV=1
M33	A0A0J5PTE1	Molecular chaperone Hsp70 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01112 PE=3 SV=1
M34	A0A0J5PV62	Inorganic diphosphatase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01699 PE=4 SV=1
M35	A0A0J5PV72	Plasma membrane ATPase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01787 PE=3 SV=1
M36	A0A0J5PV85	Eukaryotic translation initiation factor 4, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01719 PE=3 SV=1
M37	A0A0J5PVE5	Proteasome regulatory particle subunit (RpnE), putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01882 PE=4 SV=1
M38	A0A0J5PW00	Elongation factor Tu OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08056 PE=3 SV=1
M39	A0A0J5PW54	Woronin body protein HexA, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00725 PE=4 SV=1
M40	A0A0J5PW91	MFS sugar transporter OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02054 PE=3 SV=1
M41	A0A0J5PWH3	Phosphoglycerate kinase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08236 PE=3 SV=1
M42	A0A0J5PWJ7	40S ribosomal protein S0 OS=Aspergillus fumigatus Z5 OX=1437362 GN=RPS0 PE=3 SV=1

M43	A0A0J5PXI1	Conidial pigment biosynthesis 1,3,6,8-tetrahydroxynaphthalene reductase Arp2 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02124 PE=4 SV=1
M44	A0A0J5PYI6	Translation elongation factor EF-2 subunit OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02519 PE=4 SV=1
M45	A0A0J5PYP0	40S ribosomal protein S11 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02792 PE=3 SV=1
M46	A0A0J5Q035	Histone H2A OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02000 PE=3 SV=1
M47	A0A0J5Q0L0	Translation initiation factor 4B OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02236 PE=4 SV=1
M48	A0A0J5Q0U9	Prohibitin complex subunit Phb1, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05019 PE=4 SV=1
M49	A0A0J5Q217	Enolase/allergen Asp F 22 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03836 PE=3 SV=1
M50	A0A0J5Q308	Allergenic cerato-platanin Asp F13 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02605 PE=4 SV=1
M51	A0A0J5Q4K6	Dihydrolipoyl dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04772 PE=3 SV=1
M52	A0A0J5Q4L0	40S ribosomal protein S10a OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04777 PE=3 SV=1
M53	A0A0J5Q4V7	Methylmalonate-semialdehyde dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04862 PE=4 SV=1
M54	A0A0J5Q579	Spermidine synthase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05017 PE=3 SV=1
M55	A0A0J5Q5Q0	6-phosphogluconate dehydrogenase, decarboxylating OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03965 PE=3 SV=1
M56	A0A0J5SC31	Adenosine deaminase family protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_09476 PE=4 SV=1
M57	A0A0J5SDK6	Glycerol dehydrogenase (GldB), putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06531 PE=4 SV=1
M58	A0A0J5SI75	Hsp70 chaperone (HscA) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05687 PE=3 SV=1
M59	A0A0J5SNF4	Glyceraldehyde-3-phosphate dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00163 PE=3 SV=1
M60	A0A0J5SNY8	Ubiquinol-cytochrome C reductase complex core protein 2, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00378 PE=4 SV=1
M61	A0A0J5SQG5	RAS small monomeric GTPase RasA OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00948 PE=4 SV=1
M62	A0A0J5SRH5	Ubiquitin-40S ribosomal protein S27a OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01428 PE=4 SV=1
M63	A0A0J5ST40	Pyruvate dehydrogenase E1 beta subunit PdbA OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02018 PE=4 SV=1
M64	A0A0J5SW01	40S ribosomal protein Rps16 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02788 PE=3 SV=1
M65	A0A0J5SZA2	PH domain-containing protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03952 PE=4 SV=1
M66	A0A0J5T0B9	Peptidylprolyl isomerase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04347 PE=4 SV=1

M67	A0A1U8QFE6	Uncharacterized protein OS= <i>Emericella nidulans</i> (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN5441.2 PE=3 SV=1
M68	A0A1U8QSA3	40S ribosomal protein S27 OS= <i>Emericella nidulans</i> (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4777.2 PE=3 SV=1
M69	A0A1U8QSY1	Pyruvate carboxylase OS= <i>Emericella nidulans</i> (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4462.2 PE=4 SV=1
M70	A0A1U8QYP3	Uncharacterized protein OS= <i>Emericella nidulans</i> (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN5217.2 PE=4 SV=1
M71	A0A229X442	Uncharacterized protein OS= <i>Neosartorya fumigata</i> OX=746128 GN=CDV57_09631 PE=4 SV=1
M72	A0A229XRP0	Uncharacterized protein OS= <i>Neosartorya fumigata</i> OX=746128 GN=CDV57_06497 PE=4 SV=1
M73	A0A229XVC0	Uncharacterized protein OS= <i>Neosartorya fumigata</i> OX=746128 GN=CDV57_07211 PE=4 SV=1
M74	A0A229XWL9	Uncharacterized protein OS= <i>Neosartorya fumigata</i> OX=746128 GN=CDV57_05018 PE=4 SV=1
M75	A0A229XYU1	Uncharacterized protein OS= <i>Neosartorya fumigata</i> OX=746128 GN=CDV57_05509 PE=3 SV=1
M76	A1C5N9	Glyceraldehyde-3-phosphate dehydrogenase OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_004210 PE=3 SV=1
M77	A1C7X3	Translation initiation factor 4B OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_075320 PE=4 SV=1
M78	A1CAU0	Separin, putative OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_012860 PE=4 SV=1
M79	A1CDE8	Cyclin-dependent protein kinase complex component (Pcl7), putative OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_006320 PE=4 SV=1
M80	A1CDS0	Proteasome subunit alpha type OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_007560 PE=3 SV=1
M81	A1CH00	Outer mitochondrial membrane protein porin OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_046200 PE=4 SV=1
M82	A1CH47	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_046680 PE=3 SV=1
M83	A1CJD3	40S ribosomal protein S27 OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_034600 PE=3 SV=1
M84	A1CKR1	Nucleosome binding protein (Nhp6a), putative OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_039500 PE=4 SV=1
M85	A1CMJ6	Sodium/proline symporter OS= <i>Aspergillus clavatus</i> (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_097210 PE=3 SV=1

M86	A1CNV5	40S ribosomal protein S5, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_020330 PE=3 SV=1
M87	A1CQ53	S-adenosylmethionine synthase OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_024900 PE=3 SV=1
M88	A1CQS0	Coproporphyrinogen III oxidase, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_027130 PE=4 SV=1
M89	A1D007	Septin OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_039030 PE=3 SV=1
M90	A1D3G2	Uncharacterized protein OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_016510 PE=4 SV=1
M91	A1D6U2	Proteasome endopeptidase complex OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_066000 PE=3 SV=1
M92	A1DNC2	ATP citrate lyase, subunit 1, putative OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_056420 PE=4 SV=1
M93	B0XME8	GTP-binding nuclear protein OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_001470 PE=3 SV=1
M94	B0XMM5	dUTPase (Dut), putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_003270 PE=4 SV=1
M95	B0XS66	40S ribosomal protein S17, putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_026110 PE=3 SV=1
M96	B0Y239	Triosephosphate isomerase OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_061150 PE=3 SV=1
M97	B0Y5W3	Clathrin heavy chain OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_064800 PE=3 SV=1
M98	B0Y711	Cell cycle control protein (Cwf19), putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_068830 PE=4 SV=1
M99	B0Y7P8	Transcription factor TFIID complex 145 kDa subunit, putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_074560 PE=4 SV=1
M100	B0YAG9	Uncharacterized protein OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_084630 PE=4 SV=1
M101	B0YD67	Ribosomal protein L14 OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_094470 PE=4 SV=1
M102	B0YEN0	WW domain protein OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_099750 PE=4 SV=1

M103	F5AXW2	Hydrophobin (Fragment) OS=Aspergillus neoellipticus OX=91482 GN=rodA PE=3 SV=1
M104	Q58XI6	Hydrophobin (Fragment) OS=Aspergillus lentulus OX=293939 PE=3 SV=1
M105	Q5B041	Heat shock protein 60 OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=hsp60 PE=1 SV=1
M106	Q5B0X6	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN5804.2 PE=4 SV=1
M107	Q5B1U4	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN5486.2 PE=4 SV=1
M108	Q5B463	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4667.2 PE=3 SV=1
M109	Q5B948	ATP-dependent RNA helicase eIF4A OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=tif1 PE=3 SV=2
M110	Q5BEA8	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN1122.2 PE=4 SV=1

Fonte: Produção do autor.

Tabela S3. Lista combinada de proteínas identificadas em réplicas de VEs produzidas por *A. fumigatus*. ID e descrição foram obtidos pelo mecanismo de busca Mascot.

Proteína	ID	Descrição
V1	A0A0J5PDK0	CaaX prenyl protease Ste24 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06147 PE=4 SV=1
V2	A0A0J5PFT3	Ribosomal protein L14 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07318 PE=4 SV=1
V3	A0A0J5PFX3	Glutamine synthetase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07348 PE=3 SV=1
V4	A0A0J5PFZ2	GNAT family acetyltransferase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07481 PE=4 SV=1
V5	A0A0J5PFZ8	ATP synthase subunit E OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07491 PE=3 SV=1
V6	A0A0J5PGE1	Polyubiquitin (UbiD), putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06409 PE=4 SV=1
V7	A0A0J5PGJ6	5,8-linoleate diol synthase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06449 PE=4 SV=1
V8	A0A0J5PGN1	Outer mitochondrial membrane protein porin OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06063 PE=4 SV=1
V9	A0A0J5PGW6	Pyruvate carboxylase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06158 PE=4 SV=1
V10	A0A0J5PHE4	Proteasome subunit alpha type OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07220 PE=3 SV=1
V11	A0A0J5PHF7	Imidazoleglycerol-phosphate dehydratase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07230 PE=3 SV=1
V12	A0A0J5PHH4	37S ribosomal protein S9 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07245 PE=3 SV=1

V13	A0A0J5PHX6	V-type proton ATPase subunit a OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06493 PE=3 SV=1
V14	A0A0J5PI70	Proteasome subunit beta OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08484 PE=3 SV=1
V15	A0A0J5PJ23	Signal peptidase complex component OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05551 PE=4 SV=1
V16	A0A0J5PK60	Alpha/beta hydrolase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05921 PE=4 SV=1
V17	A0A0J5PKP0	Cytochrome P450 oxidoreductase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05918 PE=3 SV=1
V18	A0A0J5PL69	Amino acid permease (Gap1), putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08450 PE=4 SV=1
V19	A0A0J5PLH6	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07012 PE=4 SV=1
V20	A0A0J5PLK8	C-8 sterol isomerase (Erg-1) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06819 PE=4 SV=1
V21	A0A0J5PMJ3	Proteasome component Pup1, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03266 PE=4 SV=1
V22	A0A0J5PML5	Ribosomal protein L34 protein, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03093 PE=4 SV=1
V23	A0A0J5PMZ2	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03471 PE=4 SV=1
V24	A0A0J5PN08	Small nuclear ribonucleoprotein G OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_05584 PE=3 SV=1
V25	A0A0J5PND4	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03353 PE=3 SV=1
V26	A0A0J5PPX1	Ribosomal protein L15 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06825 PE=3 SV=1
V27	A0A0J5PPZ3	Alkaline serine protease OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03125 PE=3 SV=1
V28	A0A0J5PQ46	Peptide hydrolase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08023 PE=3 SV=1
V29	A0A0J5PSG3	Integral membrane protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00709 PE=4 SV=1
V30	A0A0J5PTJ6	Phosphatidylserine decarboxylase proenzyme 2 OS=Aspergillus fumigatus Z5 OX=1437362 GN=PSD2 PE=3 SV=1
V31	A0A0J5PTR8	Proteasome subunit alpha type OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00181 PE=3 SV=1
V32	A0A0J5PTW8	Zn-dependent hydrolase, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01259 PE=4 SV=1
V33	A0A0J5PUM3	60S ribosomal protein L8 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00486 PE=4 SV=1
V34	A0A0J5PUN7	Acyl-CoA dehydrogenase family protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00501 PE=3 SV=1
V35	A0A0J5PUW9	Altered inheritance of mitochondria protein 24, mitochondrial OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01677 PE=3 SV=1
V36	A0A0J5PUX6	Prenylcysteine lyase, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01614 PE=4 SV=1
V37	A0A0J5PVB2	Autophagic serine protease Alp2 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00761 PE=3 SV=1

V38	A0A0J5PW18	Alpha-1,2-mannosyltransferase (Kre5) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01041 PE=4 SV=1
V39	A0A0J5PWN4	60S ribosomal protein L6 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08158 PE=4 SV=1
V40	A0A0J5PWS7	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08346 PE=4 SV=1
V41	A0A0J5PX72	Lectin family integral membrane protein, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02197 PE=4 SV=1
V42	A0A0J5PX92	LEM3/CDC50 family protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01080 PE=3 SV=1
V43	A0A0J5PXA8	DNA damage response protein (Dap1) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01501 PE=3 SV=1
V44	A0A0J5PXS3	60S ribosomal protein L35a OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01691 PE=3 SV=1
V45	A0A0J5PY89	40S ribosomal protein S9 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01851 PE=3 SV=1
V46	A0A0J5PY92	Uncharacterized protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02409 PE=4 SV=1
V47	A0A0J5PYA5	Proteasome subunit alpha type OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01425 PE=3 SV=1
V48	A0A0J5PYK6	Tryptophan synthase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02549 PE=3 SV=1
V49	A0A0J5PZA0	Ribosomal protein L19 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02997 PE=3 SV=1
V50	A0A0J5PZL2	60S ribosomal protein L35 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08220 PE=3 SV=1
V51	A0A0J5PZY0	Mitogen-activated protein kinase MAF1 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04719 PE=4 SV=1
V52	A0A0J5Q0I9	Ribosomal protein OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_08104 PE=3 SV=1
V53	A0A0J5Q1V9	Proteasome subunit alpha type OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02706 PE=3 SV=1
V54	A0A0J5Q1X8	Proteasome component Prs3, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03801 PE=4 SV=1
V55	A0A0J5Q2A8	C-3 sterol dehydrogenase/C-4 decarboxylase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02365 PE=4 SV=1
V56	A0A0J5Q2N5	Proteasome component Pre2, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03988 PE=4 SV=1
V57	A0A0J5Q2W2	Proteasome subunit alpha type OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04053 PE=3 SV=1
V58	A0A0J5Q2X7	G1/S regulator NimO, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04076 PE=4 SV=1
V59	A0A0J5Q372	Transcriptional corepressor Cyc8 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02670 PE=3 SV=1
V60	A0A0J5Q3B6	ATP citrate lyase, subunit 1, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04208 PE=4 SV=1
V61	A0A0J5Q3E1	Ribosomal protein L26 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04261 PE=3 SV=1
V62	A0A0J5Q3T6	Superoxide dismutase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04913 PE=3 SV=1
V63	A0A0J5Q3U5	Inner membrane nuclease Nuc1 OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_02935 PE=4 SV=1

V64	A0A0J5Q690	Proteasome endopeptidase complex OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_03794 PE=3 SV=1
V65	A0A0J5Q6X8	Transcription factor TFIIID complex subunit, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04019 PE=4 SV=1
V66	A0A0J5Q7E7	Transmembrane 9 superfamily member OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04540 PE=3 SV=1
V67	A0A0J5SCN9	Phosphoinositide phosphatase (Sac1), putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06191 PE=4 SV=1
V68	A0A0J5SCU1	Short chain dehydrogenase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06256 PE=4 SV=1
V69	A0A0J5SE11	Tryptophanyl-tRNA synthetase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07167 PE=3 SV=1
V70	A0A0J5SE24	Import receptor subunit (Tom40) OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07187 PE=4 SV=1
V71	A0A0J5SES8	60S ribosomal protein L24a OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_07457 PE=4 SV=1
V72	A0A0J5SIH2	Catalase-peroxidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=katG PE=3 SV=1
V73	A0A0J5SJ00	Neutral/alkaline nonlysosomal ceramidase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06658 PE=4 SV=1
V74	A0A0J5SJK0	RNA polymerase I specific transcription initiation factor Rrn7, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_06878 PE=4 SV=1
V75	A0A0J5SPM8	Protein transport protein Sec61 alpha subunit OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_00658 PE=3 SV=1
V76	A0A0J5SQV8	Palmitoyl-protein thioesterase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01163 PE=4 SV=1
V77	A0A0J5SSM2	mRNA splicing protein, putative OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_01868 PE=3 SV=1
V78	A0A0J5SXM0	Fasciclin domain-containing family OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04935 PE=4 SV=1
V79	A0A0J5SZX5	Thioredoxin OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04177 PE=3 SV=1
V80	A0A0J5T0N9	Nitrilase OS=Aspergillus fumigatus Z5 OX=1437362 GN=Y699_04467 PE=4 SV=1
V81	A0A1U8QFN3	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN7475.2 PE=4 SV=1
V82	A0A1U8QI99	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN1614.2 PE=4 SV=1
V83	A0A1U8QID6	Proteasome subunit alpha type OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN6726.2 PE=3 SV=1
V84	A0A1U8QKH0	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4517.2 PE=4 SV=1
V85	A0A1U8QMK1	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN1240.2 PE=4 SV=1

V86	A0A1U8QUA3	Uncharacterized protein OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN3619.2 PE=4 SV=1
V87	A0A1U8QVB2	Catalase OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN5918.2 PE=3 SV=1
V88	A0A1U8QZA5	RL16_NEUCR 60S ribosomal protein L16 OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4202.2 PE=3 SV=1
V89	A0A229WAP8	Aldehyde dehydrogenase OS=Neosartorya fumigata OX=746128 GN=CDV57_06482 PE=3 SV=1
V90	A0A229WC49	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_05207 PE=3 SV=1
V91	A0A229WEU3	Alkaline phosphatase OS=Neosartorya fumigata OX=746128 GN=CDV57_08196 PE=3 SV=1
V92	A0A229XGB6	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_08715 PE=4 SV=1
V93	A0A229XRE2	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_07643 PE=3 SV=1
V94	A0A229XS59	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_07638 PE=4 SV=1
V95	A0A229XUN9	Beta-glucosidase OS=Neosartorya fumigata OX=746128 GN=CDV57_07025 PE=3 SV=1
V96	A0A229XV30	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_06963 PE=3 SV=1
V97	A0A229Y072	60S ribosomal protein L36 OS=Neosartorya fumigata OX=746128 GN=CDV57_04181 PE=3 SV=1
V98	A0A229Y8J6	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_01376 PE=4 SV=1
V99	A0A229YCM3	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_00390 PE=4 SV=1
V100	A0A229YCQ7	Uncharacterized protein OS=Neosartorya fumigata OX=746128 GN=CDV57_00382 PE=3 SV=1
V101	A1C3Y4	Oligosaccharyl transferase subunit (Stt3), putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_057800 PE=4 SV=1
V102	A1C6M5	60S ribosomal protein L3 OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_070790 PE=3 SV=1
V103	A1CBN5	CRAL/TRIO domain protein OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_015990 PE=4 SV=1
V104	A1CC47	Fatty acid synthase alpha subunit FasA, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_060590 PE=3 SV=1
V105	A1CCI2	Catalase OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_062020 PE=3 SV=1
V106	A1CE44	Proteasome component (Ecm29), putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_088320 PE=4 SV=1

V107	A1CEN3	Cytochrome P450 monooxygenase, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_090250 PE=4 SV=1
V108	A1CF33	Ribosomal protein L34 protein, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_091800 PE=4 SV=1
V109	A1CFC0	Uncharacterized protein OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_092670 PE=3 SV=1
V110	A1CI62	V-type proton ATPase subunit a OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_050390 PE=3 SV=1
V111	A1CII4	Copper-transporting ATPase, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_051610 PE=3 SV=1
V112	A1CIT7	Threonyl-tRNA synthetase, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_052650 PE=3 SV=1
V113	A1CL29	Signal peptidase complex catalytic subunit sec11 OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=sec11 PE=3 SV=1
V114	A1CP88	Isoleucyl-tRNA synthetase, cytoplasmic OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_021730 PE=3 SV=1
V115	A1CPP0	NADH oxidase OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_023250 PE=4 SV=1
V116	A1CSM9	Ribosomal protein L44, putative OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_080000 PE=3 SV=1
V117	A1CSQ4	Protein-S-isoprenylcysteine O-methyltransferase OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_080250 PE=3 SV=1
V118	A1CUG4	60S ribosomal protein L31e OS=Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1) OX=344612 GN=ACLA_086500 PE=4 SV=1
V119	A1CW00	Aldehyde dehydrogenase OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_102870 PE=3 SV=1
V120	A1CX29	Probable dipeptidyl peptidase 4 OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=dpp4 PE=3 SV=1
V121	A1D3Y8	Elongation factor 1-alpha OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_018320 PE=3 SV=1
V122	A1D432	Vacuolar membrane protease OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_018760 PE=3 SV=1
V123	A1D526	Uncharacterized protein OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_022300 PE=4 SV=1
V124	A1D7C0	Prenylcysteine lyase, putative OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_067810 PE=4 SV=1

V125	A1D7R3	Endo alpha-1,4 polygalactosaminidase, putative OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_069280 PE=4 SV=1
V126	A1DAL6	Catalase-peroxidase OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=katG PE=3 SV=1
V127	A1DCV5	Probable beta-glucosidase L OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=bglL PE=3 SV=1
V128	A1DFN8	ER Hsp70 chaperone BiP, putative OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_081390 PE=3 SV=1
V129	A1DFZ1	Siderochrome-iron transporter MirC OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_082450 PE=4 SV=1
V130	A1DGL5	60S ribosomal protein L10 OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_084740 PE=4 SV=1
V131	A1DGY3	Disulfide isomerase, putative OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_085950 PE=4 SV=1
V132	A1DIT8	Sorting nexin Mvp1 OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_092560 PE=4 SV=1
V133	A1DLL9	ATP synthase subunit d, mitochondrial OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_050330 PE=3 SV=1
V134	A1DNH6	Ribosomal protein L26 OS=Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / CBS 544.65 / FGSC A1164 / JCM 1740 / NRRL 181 / WB 181) OX=331117 GN=NFIA_056960 PE=3 SV=1
V135	B0XM57	Uncharacterized protein OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_001000 PE=4 SV=1
V136	B0XNP4	Ribosomal protein L15 OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_005000 PE=3 SV=1
V137	B0XQH1	60S ribosomal protein L35 OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_009940 PE=3 SV=1
V138	B0XX53	Peptide hydrolase OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_047780 PE=3 SV=1
V139	B0Y444	Isovaleryl-CoA dehydrogenase lvdA, putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_056460 PE=3 SV=1
V140	B0YBY5	Acetamidase, putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_088260 PE=4 SV=1

V141	B0YCC8	Cyclopropane-fatty-acyl-phospholipid synthase, putative OS=Neosartorya fumigata (strain CEA10 / CBS 144.89 / FGSC A1163) OX=451804 GN=AFUB_089730 PE=4 SV=1
V142	C8V7I0	Vacuolar aspartyl aminopeptidase Lap4, putative (AFU_orthologue AFUA_5G03990) OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=ANIA_11070 PE=3 SV=1
V143	C8VU50	Peroxisomal fatty acid ABC transporter, putative (Eurofung) OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=ANIA_01014 PE=4 SV=1
V144	P0CS88	NAD-dependent protein deacetylase hst2-2 OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN11873 PE=3 SV=1
V145	Q4WHM1	Proteasome component Pup1, putative OS=Neosartorya fumigata (strain ATCC MYA-4609 / Af293 / CBS 101355 / FGSC A1100) OX=330879 GN=AFUA_2G04910 PE=4 SV=1
V146	Q5B3L1	Proteasome endopeptidase complex OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4869.2 PE=3 SV=1
V147	Q5B4R3	Peptidyl-prolyl cis-trans isomerase B OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=cpr2 PE=3 SV=1
V148	Q5B5G2	Elongation factor 1-alpha OS=Emericella nidulans (strain FGSC A4 / ATCC 38163 / CBS 112.46 / NRRL 194 / M139) OX=227321 GN=AN4218.2 PE=3 SV=1

Fonte: Produção do autor.

ANEXO A

Manuscrito: BRAUER, V. S.; REZENDE, C. P.; PESSONI, A. M.; DE PAULA, R. G.; RANGAPPA, K. S.; NAYAKA, S. C.; GUPTA, V. K.; ALMEIDA, F. Antifungal Agents in Agriculture: Friends and Foes of Public Health. *Biomolecules*, v.9, p.521, 2019.

Review

Antifungal Agents in Agriculture: Friends and Foes of Public Health

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Received: 7 July 2019; Accepted: 19 September 2019; Published: 23 September 2019

Abstract: Fungal diseases have been underestimated worldwide but constitute a substantial threat to several plant and animal species as well as to public health. The increase in the global population has entailed an increase in the demand for agriculture in recent decades. Accordingly, there has been worldwide pressure to find means to improve the quality and productivity of agricultural crops. Antifungal agents have been widely used as an alternative for managing fungal diseases affecting several crops. However, the unregulated use of antifungals can jeopardize public health. Application of fungicides in agriculture should be under strict regulation to ensure the toxicological safety of commercialized foods. This review discusses the use of antifungals in agriculture worldwide, the need to develop new antifungals, and improvement of regulations regarding antifungal use.

Keywords: Antifungal agents; fungicides; agriculture; chemoinformatics

1. Introduction

There are >99,000 known species of fungi, including molds, yeasts, mushrooms, and polypores [1,2]. Fungi can grow in almost all habitats, including soil, air, seas, rivers, as well as on organic matter, including food, and other organisms, such as plants, animals, and even human skin [3]. Fungi have several significant impacts on society besides causing diseases. They are used for food production, as well as for pharmaceutical preparation, agricultural purposes, and organic-matter decomposition [4].

Among the thousands of characterized fungal species, only a few hundred are infectious and cause diseases in humans [5]. The immune system of a healthy individual has several effective mechanisms to identify, control, and eliminate fungal infections. However, in pathological conditions, including acquired immunodeficiency syndrome (AIDS), tuberculosis, diabetes mellitus, and cancer, or under increased physiological stress, such as during organ transplantation, corticosteroid administration, and chemotherapy [6,7], the risk of developing fungal infections-related ailments is highly increased [8]. Among the infectious fungal species, *Aspergillus* spp., *Candida* spp., *Cryptococcus* spp., and *Pneumocystis jirovecii* are the causative agents of major mycoses in humans [9,10]. In addition, the incidence of

infections caused by *Zygomycetes*, *Fusarium*, and *Scedosporium* has been rising [11,12]. Recent global estimates have revealed approximately 3,000,000, 250,000, 700,000, and 223,100 cases of chronic pulmonary aspergillosis, invasive aspergillosis, invasive candidiasis, and cryptococcal meningitis among AIDS patients [13]. Moreover, the mortality rates associated with invasive fungal infections are >50% even with antifungal treatment, possibly because of the late diagnosis of the infections and identification of the causative fungi [3,11].

It is difficult to develop novel fungicides with ideal characteristics, including broad-spectrum effectiveness, enhanced bioavailability, and minimal toxicity and side effects, due to similarities between fungal and mammalian cells, such as in the biosynthetic pathways and chromatin organization of DNA [6,14]. Consequently, drug development against invasive fungal pathogens has been slow. It began in the 1950s, with the approval of polyene amphotericin B deoxycholate, which was followed by the development of the pyrimidine analog flucytosine in the 1960s, azoles in the 1970s, and echinocandins in the 2000s [15]. Fungi have plastic genomes and reproduce rapidly [16]. In addition to these properties, the increased usage of prophylactic antifungal agents and empirical and directed therapies has increased the number of drug-resistant pathogenic fungal strains [17]. Moreover, antifungal drugs used in agriculture can serve as environmental drivers for the development of drug-resistant fungal strains [18]. To decrease the development of drug resistance of fungi in fields, the use of a mix of antifungals with different action mechanisms is encouraged [16,17]. Thus, the aim of this review is to discuss the use of antifungal agents in agriculture, its correlation with the development of drug-resistant fungal strains, as well as the consequences of unregulated antifungal use on public health.

2. Agricultural Fungicides

Agricultural pesticides are chemicals that are used to kill crop pests or inhibit the growth or harmful effects of these organisms [19]. Among the different classes of pesticides, fungicides include physical, chemical, or biological agents intended to combat fungal microorganisms [20]. These are widely used in agricultural systems to control diseases and preserve the yield and quality of crops [21]. The history of the agricultural sector is shaped by constant challenges with respect to increasing productivity and, thus, supply to meet the increasing need for consumption, and, thus, demand [20]. The increased need for productivity has mainly been addressed by the eradication of pests through the use of pesticides [22]. According to the Food and Agriculture Organization of the United Nations (FAOSTAT), the main crops produced in the world between 2013 and 2017 were cereals (especially coarse grains, maize, rice, and wheat), sugarcane, primary vegetables and fruits, and plants grown for their roots and tubers (Figure 1) [23]. During this period, the African continent was the main producer of cereals and roots/tubers, for which the yearly average production was 5 M tons (80.4%) and approximately 7 M tons (71.7%), respectively. Asia's main agricultural product during the same period was rice, with a yearly average production of 668 M tons, corresponding to 90.4%. China, India, and Indonesia showed the highest agricultural productivity among Asian countries [23]. The American continents had a high production of soybeans (88.2%), sugarcane (54.8%), and maize (50.4%). For instance, on average, the United States alone produced 363 and 108 M tons of maize and soybeans, respectively, per year. Brazil's main agricultural product during this period was sugarcane, with a yearly average production of 756 M tons. Additionally, it was the second and third largest producer of soybeans and maize, respectively [23].

Figure 1. The annual global production of major crops during 2013–2017 (Source: FAOSTAT. www.fao.org. Access on: 06 November 2018).

Fungal phytopathogens affecting agricultural crops lead to a decrease in their quality and production [24]. They act as a threat to crops [25] through various mechanisms of pathogenesis that compromise the immune system of the plants (Table 1) [24]. The use of fungicides against fungal plant diseases improves crop yield, quality, and shelf-life [21,26]. Some examples of antifungal agents include benzimidazoles, dithiocarbamates, strobilurins, and azoles [26], with azoles, especially triazoles, being widely used in fields [27].

Table 1. Some important fungal pathogens and their associated diseases in agricultural crops.

Fungal Pathogen	Crops	Disease	Crop Loss (%)
<i>Botrytis cinerea</i>	Fruits and ornamental flowers [28]	Gray mold [28]	Up to 30% to 40% loss of strawberries [29]
<i>Blumeria graminis</i>	Wheat and barley [28]	Mildews of grasses [28]	18% potential and 13% <i>de facto</i> loss of grains under current disease control [30]
<i>Colletotrichum</i> spp.	Fruits and vegetables [28]	Anthraxnose spots and blights [28]	Losses >80% in tropical, sub-tropical and Mediterranean regions [31]
<i>Cladosporium fulvum</i>	Tomato [32]	Tomato leaf mold [32]	Loss of 10–25% during regular years [33]
<i>Fusarium</i> spp.	Potato [34]	Dry rot of tubers [34]	Crop losses of up to 25%. During storage, >60% of tubers can be infected [35]
<i>Fusarium graminearum</i>	Cereals [28]	Fusarium head blight; Fusarium ear blight or head scab [28]	In China, 5–10% loss. In Europe and South America, up to 50–60% and 70% of loss [30]
<i>Magnaporthe oryzae</i>	Rice [28]	Rice blast [28]	Losses vary between 10% and 35% depending on the variety and environmental conditions [25]
<i>Mycosphaerella graminicola</i>	Wheat [28]	Septoria tritici blotch [28]	Up to 30–50% loss [25]
<i>Puccinia</i> spp.		Rust [28]	70% loss [36]
<i>Phakopsora pachyrhizi</i>	Soybean [36]	Rust [36]	Up to 70% loss [36]
<i>Pytophthora infestans</i>	Potato [34]	Late blight [34]	16% loss [34]
<i>Rhizoctonia solani</i>		Stem canker and black scurf [34]	30% loss [34]
<i>Sporisorium scitamineum</i>	Sugarcane [37]	Sugarcane smut [37]	Up to 62% loss [38]
<i>Ustilago maydis</i>	Corn [28]	Corn smut [28]	Up to 20% loss [36]

The first compound with fungicidal properties was described by Bénédict Prévost in 1807 when he found that germination of spores from *Tilletia caries* was inhibited by pieces of metallic copper placed in the soil [39]. At the beginning of the 20th century, the first organic fungicide, an organomercurial compound, was synthesized. Further studies in this direction resulted in the commercialization of several fungicides, such as 2-methoxyethyl silicate and 2-hydroxyphenyl mercury, which are effective against fungi, such as *Fusarium* spp. and *Dreschlera* spp. [40].

In recent years, the agricultural sector has faced several challenges involving decreased crop yield due to pests, diseases, and abiotic stresses [41]. In addition, the global population is estimated to grow by approximately 30% by 2050, necessitating investments to increase agricultural production and productivity [23]. Therefore, the use of efficient fungicides for protection of agricultural crops from disease during both large-scale agricultural production and post-harvest stages is necessary [42].

The global pesticide use increased significantly during 2012–2016, with a peak observed in 2014. During this period, Asia showed the highest pesticide use, totaling 2 M tons (52%) of average pesticide use, followed by America with an average use of 1 M tons (32.7%), Europe with 477 K tons (11.6%), Africa with 95 K tons (2.3%), and Oceania with approximately 55 K tons (1.4%) (Figure 2). Brazil used the highest amounts of fungicides [23], followed by Italy, Spain, France, Colombia, United States of America, Mexico, Japan, Turkey, and Ukraine [23]. Data from the National Union of the Product Industry for Plant Protection (SINDIVEG) showed that, in 2016, fungicides became the most commercialized product category in Brazil, accounting for 33% of the total market [43].

Figure 2. Use of pesticides in the world during 2012–2016 (Source: FAOSTAT. www.fao.org. Access on: 17 December 2018).

2.1. The Use of Antifungal Agents in Agriculture Poses a Potential Threat to Human Health

Different scientific studies conducted in the late 1950s and early 1960s showed that agricultural pesticides might threaten human health in the long term [44]. These threats derive from the exposure of consumers and workers to pesticides through contact, inhalation, or ingestion of food or water contaminated with pesticides [20]. Adverse effects of such exposures have been reported and include endocrine, immunological, neurological, and carcinogenic problems, as well as premature births [44].

In addition to health problems, the excessive use of pesticides can also cause environmental problems; accumulation of pesticides in the environment disrupts the ecological balance and gives rise to pathogenic resistance to the pesticides [45,46]. Thus, the application of fungicides in agriculture should be under strict regulation to ensure that commercialized foods are safe for consumption and pose negligible risks of acute toxicity due to carry-over [42].

2.2. Antifungal Resistance

Antifungal resistance is a heritable fungal characteristic that develops through natural selection of fungi [47]. The selective pressure exerted on fungi by exposure to a fungicide “selects” one or more strains that exhibit resistance to that fungicide or have a “fungicide-resistant phenotype” [48,49]. These strains can, then, survive and reproduce in the presence of this fungicide [48]. Biological factors that enable the spread of a fungicide-resistant fungal pathogen include a short life cycle, abundant sporulation, and long-distance spore dispersal [50]. In addition to these factors, which are intrinsic to the fungal species, development of fungicide resistance depends on how the fungicide is used [51]. There are four major mechanisms of fungal resistance development: (i) alterations in the target protein due to mutations, (ii) upregulation of the target protein, (iii) decrease in drug effective concentration, mainly because of development of efflux processes, and (iv) detoxification by metabolic enzymes, resulting in the degradation of the fungicide [14,21,52]. The possible mechanisms of resistance development against various antifungal drugs are summarized in Table 2 [16]. The most important and frequently used antifungals in agriculture are discussed below.

Table 2. The main classes of antifungal drugs and the mechanisms of development of resistance to them.

Antifungal Class	Mechanism of Action	Examples of Antifungal Drugs	Examples of Resistant Fungal Species	Mechanism of Resistance
Methyl benzimidazole carbamate	Inhibits microtubule assembly [53]	Benomyl, carbendazim, flubendazole [54]	<i>Botrytis cinerea</i> , <i>Venturia inaequalis</i> [55,56]	Point mutation in β -tubulin gene [56,57]
Succinate dehydrogenase inhibitor	Inhibition of fungal respiration by binding to the ubiquinone-binding site in the complex II of mitochondria [58]	Carboxin, benodanil, flutolanil, fenfuran, fluxapyroxad, thifluzamide, furametpyr [59]	<i>Botrytis cinerea</i> , <i>Alternaria alternata</i> , <i>Didymella brioniae</i> , <i>Podosphaera xanthii</i> , <i>Corynespora cassicola</i> [60–63]	Mutations in succinate dehydrogenase gene (amino acid substitution H257L or H257Y) [58,62]
Anilinopyrimidine	Inhibition of methionine synthesis and secretion of hydrolytic enzymes [64]	Cyprodinil, mepanipyrim and pyrimethanil [65]	<i>Botrytis cinerea</i> , <i>Venturia inaequalis</i> , <i>Oculimacula</i> spp. [66–68]	This mechanism is not completely clear; it has been suggested to involve the overproduction of ABC (ATP-binding cassette) transporters or the modification of the target sites [69]
Qo inhibitor	Blocks fungal energy production through inhibition of mitochondrial respiration by binding to the Qo site of complex III [70]	Azoxystrobin, mandestrobin, pyraclostrobin, kresoxim-methyl, dimoxystrobin, famoxadone, fluoxastrobin, fenamidone, pyribencarb [65]	<i>Erysiphe necator</i> , <i>Pseudoperonospora cubensis</i> , <i>Venturia inaequalis</i> , <i>Alternaria solani</i> , <i>Pyrenophora teres</i> , <i>Pythium aphanidermatum</i> , <i>Pyrenophora tritici-repentis</i> [65]	Point mutations in the mitochondrial cytochrome b (<i>cyt b</i>) gene (G143A, F129L, G137R) [57,70]
Morpholine	Inhibition of ergosterol synthesis by blocking $\Delta 14$ -reductase and $\Delta 8$ - $\Delta 7$ -isomerase [6]	Aldomorph, fenpropimorph, dodemorph, tridemorph [65]	Decreased sensitivity in powdery mildews [16]	Unknown [16]
Azole	Suppression of ergosterol synthesis by inhibiting 14 α -demethylase [12]	Imazalil, oxpoconazole, triflumizole, diniconazole, epoxiconazole, flutriafol [65]	<i>Zymoseptoria tritici</i> , <i>Venturia inaequalis</i> , <i>Penicillium digitatum</i> , <i>Cercospora beticola</i> , <i>Monilinia fructicola</i> , <i>Brumeriella jaapii</i> , <i>Botrytis cinerea</i> , <i>Penicillium digitatum</i> , <i>Zymoseptoria tritici</i> [71,72]	Mutations in <i>cyp51</i> , upregulation of <i>cyp51</i> and the genes encoding membrane transporters [72]

2.2.1. Methyl Benzimidazole Carbamate (MBC)

MBC or Benzimidazole, is a heterocyclic compound and a benzo derivate of imidazole [73]. Described in 1969, it exhibits several biological activities, such as anti-parasitic, anti-helminthic, anti-viral, and anti-neoplastic activities among others [74]. Since its discovery, benzimidazole has been widely used for crop management [75] as an innovative fungicide with a systemic curative activity that allows for longer intervals between consecutive sprays [65]. This antifungal agent inhibits microtubule assembly, mainly by binding to the free β -tubulin monomers in the colchicine-binding site [53,76]. Suppression of microtubule formation, in turn, impairs cell division and may lead to cell death [75].

Problems regarding resistance to benzimidazole emerged soon after it was introduced into the market. First observed in *Botrytis* spp. in 1971, benzimidazole resistance has been reported in approximately 115 species of fungi to date [77]. The mechanism of resistance to this class of fungicides involves generation of point mutations in the β -tubulin gene [57]. The first report of such a mutation was published in 1992 by Koenraadt et al. who showed a conversion in codon 198 of the *Venturia inaequalis* β -tubulin. This codon encodes alanine in the β -tubulin protein of the ordinary strain, glutamic acid in an MBC-sensitive strain, lysine in a highly MBC-resistant strain, and glycine in a moderately MBC-resistant strain. Another mutation was reported in codon 200 of a medium resistant strain, which generates phenylalanine instead of tyrosine [56]. In addition, mutations in codons 6, 50, 167, and 240, which may lead to benzimidazole resistance, have been reported in field isolates [57].

2.2.2. Succinate Dehydrogenase Inhibitor (SDHI)

Carboxin, a generation I SDHI, was the first fungicide of this class. It was introduced into the market in 1966 [59] and targets basidiomycete pathogens [21]. Generation II SDHIs, including boscalid, fluxapyroxad, and fluxypyrim, exhibit high antifungal activity in cereals, fruit trees, vegetables, and field crops [62]. This class of antifungals inhibits fungal respiration by blocking the ubiquinone-binding site (Q-site) in complex II of mitochondria [61].

SDHIs show a broad-spectrum activity, but their intensive application likely causes selective pressure leading to the development of resistant pathogen strains [59]. Since this class of fungicides includes single-site inhibitors, the FRAC (Fungicide Resistance Action Committee) classified them as posing medium–high risk for emergence of resistant strains [65]. Interestingly, there are reports of an association between carboxin and boscalid resistance and mutations in the succinate dehydrogenase gene [62]. The most common mutations involved are the substitutions H257L and H257Y, which replace the histidine residue at position 257 (a ubiquinone-binding site) of the succinate dehydrogenase gene with leucine and tyrosine, respectively [58,62]. *Botrytis cinerea*, *Alternaria alternate*, *Didymella brioniae*, *Podosphaera xanthii*, and *Corynespora cassiicola* are examples of fungi that have been reported to be resistant to SHDI [62].

2.2.3. Anilinopyrimidine (AP)

Anilinopyrimidine (AP) was introduced into the market between 1992 and 1995 and is used against ascomycetes [68]. Cyprodinil, mepanipyrim, and pyrimethanil belong to this fungicide group [65]. This class of fungicides is used to control gray mold caused by *B. cinerea* in fruits, vegetables, and ornamental flowers and also to control apple scab caused by *Venturia inaequalis* [68].

The action mechanism of AP involves the inhibition of methionine synthesis and hydrolytic enzyme secretion (proteases, cellulases, cutinases, and lipases) [64]. It is considered to pose a moderate risk of resistance development [65]. AP resistance has been reported in *B. cinerea*, *V. inaequalis*, and *Oculimacula* spp. [51]. Among these, AP-resistant *B. cinerea* strains have been reported in several European vineyards [55]. Low concentrations of AP were used to inhibit the tube-elongation and mycelial growth of wild-type *B. cinerea* strains. However, the AP-resistant *B. cinerea* strains Ani R1, Ani R2, and Ani R3 have emerged with time. These strains exhibit a significant degree of resistance to AP, with Ani R1 showing moderate to high resistance at all stages and Ani R2 and Ani R3 showing

resistance only in the germ-tube elongation stage [55]. The possible mechanism through which strains Ani R2 and Ani R3 resist AP activity might be the energy-dependent efflux (ABC-transporter) of the fungicide [69]. Mutations at the target sites of AP in Ani R1 have been reported [69]; however, more studies are needed to identify the molecular mechanisms underlying AP-resistance in this strain [68,69].

2.2.4. Qo Inhibitor (QoI)

QoI is also called “strobilurin” because this class of fungicides was derived from a natural compound called strobilurin A produced by mushrooms (basidiomycetes) of the genera *Strobilurus* [78,79]. This class of natural compounds is unstable in the presence of light and, thus, is not useful for the management of crop diseases [79]. However, modifications introduced into the chemical structure of one of these compounds generated a photo-stable version with antifungal activity, allowing strobilurin to be introduced into the market in 1996 [78,79]. Currently, there are 18 fungicides of this class, with different chemical groups in their structure, available on the market, including methoxyacrylates, methoxyacetamide, methoxycarbamates, oximinoacetates, oximinoacetamides, axazolidinedones, dihydrodioxazines, imidazolinones, and bezylcarbarnates [51,65], but they all share a common mechanism of action.

The name “QoI” arose because this fungicide class inhibits binding at the Qo (quinol oxidation) site of complex III (cytochrome bc₁ enzyme complex) during mitochondrial respiration [80]. Thus, the electron transfer between cytochrome b and c does not occur, blocking NADPH (nicotinamide adenine dinucleotide) oxidation and ATP (adenosine triphosphate) production [79,81]. QoIs exhibit fast action since the lack of energy in fungal cells affects the spore germination process and zoospore motility [80]. This class has a broad-spectrum activity against fungi, including ascomycetes, basidiomycetes, and oomycetes [65], and is used for treatment of several crops infected with these fungi. Despite these features, QoI is classified by FRAC as a fungicide class with high risk for the development of fungal resistance [51]. The main mechanism of fungal resistance related to this class involves point mutations in the mitochondrial cytochrome b (*cyt b*) gene [57] that result in changes in the amino acid sequence of the protein, preventing fungicide binding to it [80]. The following three-point mutations have been described as the cause of development of resistant phenotypes: substitution of alanine for glycine at position 143 (G143A), leucine for phenylalanine at position 129 (F129L), and arginine for glycine at position 137 (G137R) [70]. These three mutations lead to different degrees of resistance against QoIs; G143A is associated with high resistance and F129L and G137R are associated with moderate resistance [70]. At least 20 pathogens have been reported to have resistance against QoIs around the world [82]. Among the described QoI-resistant fungi, *Erysiphe necator*, *Pseudoperonospora cubensis*, and *V. inaequalis* carry the G143A mutation [83], whereas *Alternaria solani*, *Pyrenophora teres*, and *Pythium aphanidermatum* carry the F129L mutation, and *Pyrenophora tritici-repentis* carries the G137R mutation [65].

2.2.5. Morpholine

Morpholine is an organic compound with a heterocyclic ring containing oxygen and nitrogen and has various biological effects, such as anti-parasitic, anti-cancer, anti-inflammatory, anti-malarial, and anti-fungal effects [84]. Its antifungal action was described in 1965, and dodemorph, tridemorph, aldimorph, and fenpropimorph are some of the members of this antifungal class [65].

Morpholines are systemic fungicides used to control powdery mildews and cereal foliar diseases [85]. The class exerts its antifungal activity via the inhibition of two enzymes involved in ergosterol synthesis: $\Delta 14$ -reductase and $\Delta 8$ - $\Delta 7$ -isomerase [6]. The FRAC classifies morpholines as posing a moderate risk for resistance development [51]. Although there have been reports of decreased sensitivity to this class of fungicides in powdery mildews, the mechanism underlying this resistance remains unknown [16].

2.3. Azole Resistance of *Aspergillus*: Implications in Clinic and Fields

Alterations in target proteins have been demonstrated for several fungicides, including azoles, the main antifungal class used for crops [16]. Azoles are generally sprayed in fields to control rust and mildew affecting fruits, vegetables, cereal, and other crops [86,87]. Azoles have a synthetic origin and a cyclic structure like imidazoles and triazoles [12]. Their mechanism of action involves interfering with the enzymatic activity of lanosterol 14 α -demethylase (also known as CYP51), a member of the P450 enzyme family. This enzyme converts lanosterol to ergosterol, which is an essential component of the fungal cell membrane and contributes to its fluidity and integrity as well as the efficient functioning of membrane-bound enzymes. Inhibition of CYP51 activity results in the accumulation of demethylated lanosterol at toxic rates, disturbing the dynamics and stability of the cell wall. Consequently, the fungal growth and replication become suppressed [12,26,27,88]. Azoles are extensively used, since they are inexpensive, have a broad spectrum action, and are effective against plant fungal diseases. In addition, these fungicides are used in grain and grass environments during pre- and post-harvest periods to prevent contamination by yeast (such as *Candida* spp., *Trichosporon penicillatum*, and *Cryptococcus* spp.) and filamentous fungi (such as *Aspergillus* spp., *Fusarium* spp., and *Alternaria* spp.) [87]. However, the excessive use of azoles leads to contamination of soil, air, and plants, mainly because of their lipophilic characteristic, which results in their absorption into soil and organic matter. Azoles exhibit high stability, and can remain virtually unchanged in the environment and in food for months [87,89].

Fungal resistance to azoles could be due to various factors. Mutations in *cyp51A* can reduce the affinity of the encoded protein to its inhibitors and upregulation of this gene increases azole efflux by upregulation of membrane transporters [72]. In field isolates, the most common reason for azole-resistance has been found to be mutations in *cyp51A* [71,72]. *Zymoseptoria tritici* harbors >30 modifications in *cyp51A* [71]. Species, such as *V. inaequalis*, *Penicillium digitatum*, *Cercospora beticola*, *Monilinia fructicola*, and *Blumeriella jaapii*, are associated with azole-resistance caused by *cyp51A* upregulation resulting from insertions of variable sizes in the gene promoter [72]. Efflux transporters, such as ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters, can reduce the azole concentration in fungal cells. Among the field isolates of fungi, this type of resistance has been reported for *B. cinerea*, *Penicillium digitatum*, and *Zymoseptoria tritici* [71].

The extensive use of azoles in agriculture can affect phytopathogens with medical relevance [26]. Consequently, fungi causing important human mycoses may also develop azole-resistance [26,87]. Several human diseases are caused by fungi that survive in various environments and foods, such as *Coccidioides*, *Histoplasma*, *Aspergillus*, and *Cryptococcus* [87]. *Aspergillus fumigatus*, a saprophytic fungus that can live in soil, produces spores that are airborne and can be inhaled by humans. The acquired resistance of *A. fumigatus* against commonly used antifungal drugs might be due to the extensive use of fungicides [90,91]. In the clinic, *A. fumigatus* resistance has been observed in patients who received long-term azole therapy against aspergillosis, mainly because azoles are the first choice of drugs used for the treatment of this fungal disease [92]. However, several cases of *A. fumigatus* resistance occurred in patients who were never treated with azoles; thus, it has been hypothesized that there could be other sources of exposure to fungi with acquired resistance against azoles, such as agricultural crops [93].

Snelders et al. (2008) reported such an association between *A. fumigatus* azole resistance and environmental exposure [94]. They investigated the prevalence of intraconazole resistance in 1912 clinical isolates of *A. fumigatus* collected from 1219 patients at the University Medical Centre in Nijmegen during a period of 12 years and compared them with the clinical isolates from hospitals in different cities. They confirmed that there was a wide-spread azole resistance among the intraconazole-treated samples, with 94% of the resistant isolates carrying *cyp51A* mutations. Since person-to-person transmission of *Aspergillus* infection is not common, a similar TR₃₄/L98H mutation in unrelated patients indicated the involvement of environmental factors. For instance, it is possible that the modified conidia were dispersed by the air and, consequently, caused the infection [94].

The main mechanism underlying azole resistance acquired by *A. fumigatus* involves point mutations in or upregulation of *cyp51A* (14 α -demethylase in *A. fumigatus*) [95]. Several point mutations

in *cyp51a*, such as G54, M220, G448S, G138, P216, and F219, have been reported to confer azole resistance onto *A. fumigatus* [93,96]. In addition, polymorphisms that lead to amino acid mutations, such as F46Y, M172V, N248T, D255E, and E427K, have already been associated with the azole resistance of *A. fumigatus* [95]. Tandem insertions (46-bp long in total) in the *cyp51a* promoter region and substitution of tyrosine 121 to phenylalanine and threonine 289 to alanine (TR₄₆/Y121F/T289A) were found to be associated with voriconazole resistance. In addition, a 53-base pair sequence inserted in tandem in the promoter region has been correlated with resistance to azoles. The mutation TR₃₄/L98H, which results in the overexpression of *cyp51A*, is the main mutation associated with *A. fumigatus* resistance to azoles [94,95]. Azole resistance among clinical isolates of *Aspergillus fumigatus* has been demonstrated in a recent study [97]. In this report, three isolates with itraconazole resistance carried diverse *cyp51A* mutations. One of these isolates harbored the mutation M220K, while a second exhibited the G54 mutation in addition to a modification in the *cyp51A* promoter. The third isolate had an integration of a 34-bp tandem repeat (TR34) in the promoter region of the gene and an L98H substitution (substitution of leucine 98 with histidine).

The phenomenon of azole-resistance of *A. fumigatus* is widespread and it has been reported in Middle East, Asia, Africa, Australia, North Europe, and South America [98]. It is not yet clear where or when this mechanism of resistance initiated; however, it has been suggested that it is probably due to a common ancestral gene, since there is lower genetic diversity among unrelated *A. fumigatus* strains [98]. It is important to highlight that approximately 70% of patients with azole-resistant aspergillosis had not undergone any azole treatment at all [98,99]. Meireles et al. [100] evaluated the change in the clinical antifungal sensitivity of *Aspergillus flavus* in response to azole and benzimidazole fungicides [100]. They showed that exposing *Aspergillus flavus* to azoles changed the sensitivity of the fungus to the antifungals itraconazole, voriconazole, and posaconazole, evidencing the development of resistant phenotypes, and constituting the first case report of antifungal resistance induced by azole exposure. Dos Reis et al. [101] investigated the *Aspergillus fumigatus* MSH2 mismatch repair (MMR) gene *mshA* and its impact on virulence and evolution of azole resistance [101]. The *mshA* mutant *A. fumigatus* strain showed significantly reduced virulence in a neutropenic murine model of invasive pulmonary aspergillosis. In addition, the mutant strain exhibited a rapid acquisition of virulence and high levels of resistance to posaconazole.

In this context, public health surveillance programs for fungal diseases must be put in practice because the excessive use of azole in the field is already causing harm in clinical settings [94]. Azoles are the first line of treatment against *Aspergillus* infection. As diseases caused by this fungal species, including invasive aspergillosis, allergic manifestations, and chronic pulmonary disease, can affect a large number of people, fungal acquisition of azole resistance will lead to increased mortality due to *Aspergillus* infections around the world [17,102].

3. New Antifungal Strategies

Chemical control is essential for the maintenance of reliable and good crop yields [103]. To conserve fungicides available in the market and to protect the new arrivals, further research along with increased cooperation between industries and regulatory agencies is required [51]. However, the search for new fungicides has been challenging due to increased resistance to fungal pathogens [104]. Awareness regarding environmental safety has generated public demand for effective and safe antifungal biocontrol agents that are economically, environmentally, and socially sustainable [105]. Nanotechnology has emerged as a new research area in the present century and allows the use of nanoparticles and nanomaterials for protection of agricultural crops against fungal pathogens [106]. Copper nanoparticles have received increasing attention in this regard because their antimicrobial activity has been known since ancient times [107,108]. Copper was used in agriculture in 1761 for the first time, when it was observed that soaking seed grains in a weak solution of copper sulfate inhibited the growth of fungi present in the seeds [109]. Since then, copper compounds have been widely used in agricultural practices, such as for antifungal [110] or antimicrobial purposes [111,112].

Kanhed et al. showed that copper nanoparticles exhibit promising antifungal activity against phytopathogenic fungi. They also observed that these nanoparticles exhibited better antifungal activity than that of bavistin, which is a commercially used fungicide. Therefore, copper nanoparticles might be used in agriculture as a novel antifungal strategy for the control of fungal pathogens of plants [106].

Besides metallic nanoparticles, chitosan nanoparticles have been proposed as potential biopesticides against fungal infections [113]. The antifungal activity of nanoparticles in agriculture is poorly studied; however, some studies have focused on the use of low concentrations of nanoparticles with low toxicity in the agricultural sector [114]. Servin et al. (2015), and Ditta and Arshad (2016) demonstrated that nanomaterials can suppress plant diseases, increase agricultural yield, and provide more nutrients to plants than fertilizers [115,116].

Several plants have been used for therapeutic and prophylactic treatment against several infectious diseases since ancient times. Some good, natural-based fungicides have showed promising antifungal activity and should be a good alternative to combat fungal pathogens in agriculture. Volatile constituents from *Origanum onites* have been evaluated regarding to their antifungal activity against the pathogens *Phomopsis obscurans*, *Fusarium oxysporum*, *Colletotrichum species*, and *Botrytis cinerea* [117]. Thus, a better understanding of the natural based fungicide strategies and its impact on plant-fungal interactions could decrease the use of pesticides in agriculture.

Recently, Hao et al. (2017) analyzed the antifungal activities of ferric oxide (Fe_2O_3), copper oxide (CuO), and titanium oxide (TiO_2) nanoparticles and three carbon nanomaterials, multi-walled carbon nanotubes (MWCNTs), fullerene (C_{60}), and reduced graphene oxide (rGO), against the fungus *B. cinerea* [114]. This fungus damages fruits, vegetables, and ornamental plants [118]. In plants, such as roses, it causes gray mold, which results in a loss of approximately 30% plant productivity per year [119]. Through in vitro and in vivo experiments, Hao et al. (2017) found that each of the three aforementioned carbon nanomaterials inhibited *B. cinerea* infection in roses at concentrations of 200 mg/L. This inhibitory effect derives from the interaction between the carbon nanomaterials and fungal spores, leading to the aggregation of the spores and, thus, suppressing their germination. As with the Fe_2O_3 and CuO nanoparticles, carbon nanomaterials showed significant antifungal effects even at concentrations of 50 mg/L. While TiO_2 nanoparticles exhibited no evident effects, there was a decrease in the number of micelles formed by them relative to the control. Therefore, this study demonstrated the antifungal activity of carbon nanomaterials and metal nanoparticles in *B. cinerea* and emphasized that the application of nanoparticles should be carefully evaluated because of their potential toxicity and environmental risks [114].

To date, various strategies have been developed to reduce antifungal resistance in agricultural settings while promoting the development of new antifungal agents [16]. Currently, combinatorial fungicide treatment constitutes one of the strategies used to delay antifungal resistance [120,121]. In addition, research in the areas of synthetic biology and epigenomics has allowed the development of new antifungal agents based on RNA interference approaches [122], such as bi-directional trafficking of plant-fungal miRNAs, for the control of pathogens, including *B. cinerea* [123]. Such approaches for the development of new antifungal strategies are promising and potentially transformative [16].

3.1. Endophytic Fungi in Agriculture

In recent decades, the search for alternatives for disease control in agriculture has been gaining prominence because some fungal pathogens have gained antifungal resistance and some synthetic chemicals have been banned for being pollutive and toxic [124]. Recent advances have shown that the use of microorganisms, mainly bacteria and fungi, as biological control agents has been advantageous for the control of diseases and pests, improving agricultural yields [125,126].

Endophytic fungi have been studied as an alternative and sustainable means of converting the natural compounds in host plants to antifungal compounds, which are not only effective against human pathogens but also against phytopathogens [127]. Endophytic microorganisms are found in plant species of extreme importance [124] and can be classified as competent, optional, obligatory,

opportunistic, or passive, depending on their effects on plants [128–131]. Recently, they have gained special attention due to the benefits these microorganisms can confer onto their hosts in the form of pesticides, helping the growth and survival of plants and increasing their tolerance to extreme temperatures and drought as well as removing contaminants from the soil [132]. *Trichoderma* spp. are associated with the soil and include some important species, such as *T. hamatum*, *T. harzianum*, *T. polysporum*, and *T. viride*, which are important fungal biocontrol agents in plants [133,134]. Many species of *Trichoderma* are used to combat soil fungal pathogens and some follicular pathogens [135,136]. The principal advantages of this type of biocontrol agents are that they easily adapt to various environmental conditions, exhibit tolerance to certain fungicides, have diverse mechanisms of action and simple nutritional requirements, and grow fast [124,137].

Another beneficial approach involving endophytic fungi makes use of their production of bioactive secondary metabolites, which represent a group of microorganisms capable of synthesizing new compounds [138] that can target plant pathogens and pests [139]. Deshmukh et al. (2018) reported several metabolites produced by endophytic fungi from medicinal plants and their potential as antifungal agents [127]. For example, scleroderma A and B and triterpenoid lanostane are produced by an endophyte basidiomycete fungus associated with the *Eucalyptus grandis* plant and these compounds were identified as potential fungicides against *Candida albicans*, *C. tropicalis*, *C. crusei*, and *C. parapsiosis*. Scleroderma B showed better antifungal activity against all these fungal species than scleroderma A or triterpenoid lanostane [140].

Xylaria spp., associated with the *Azadirachta indica* plant from China, are a source of new bioactive compounds, some of which exhibit relevant pharmacological properties for drug discovery [141], presenting with antifungal activities against *C. albicans*, *Aspergillus niger*, and *Fusarium avenaceum* [127,142]. The fungus *Mycosphaerella*, which is an endophyte of the plant *Eugenia bimarginata* from Brazil, has been shown to produce two eicosanoid acids that have antifungal activities against *C. neoformans* and *C. gattii* [143]. The cryptocandin lipopeptide is isolated from the fungus *Cryptosporiopsis quercina*, which grows in wood species in Europe. This compound is active against several fungi pathogenic against plants, including the fungal species *B. cinerea* [144], and is related to some antimycotic compounds, such as echinocandins and pneumocandins (Table 3) [145].

Table 3. Production of secondary metabolites by endophytic fungi and their potential as antifungal agents.

Endophytic Fungi	Plant	Metabolites	Antifungal Activities
Basidiomycete fungus [140]	<i>Eucalyptus grandis</i> [140]	Scleroderma A and B Triterpenoid lanostane [140]	<i>Candida albicans</i> <i>C. tropicalis</i> <i>C. grusei</i> <i>C. parapsiosis</i> [140] <i>C. albicans</i>
<i>Xylaria</i> spp. [141]	<i>Azadirachta indica</i> [127,142]	Bioactive compounds [141]	<i>Aspergillus niger</i> <i>Fusarium avenaceum</i> [127,142]
<i>Mycosphaerella</i> spp. [143]	<i>Eugenia bimarginata</i> [143]	Eicosanoid acids [143]	<i>Cryptococcus neoformans</i> <i>C. gattii</i> [143]
<i>Cryptosporiopsis quercina</i> [144]	Wood species in Europe [144]	Cryptocandin lipopeptide [144]	<i>Botrytis cinerea</i> [144]

Novel molecular biology approaches have been used for the identification and characterization of genetic elements and metabolites involved in the interactions between plants and endophytic microorganisms [146]. For these endophytic microorganisms to be marketed as successful biocontrol agents, certain criteria must be met regarding their ease of application and dissemination to crops without causing any off-target effects. Such microbes present with a wide range of effective modes of action and, under no circumstance, cause symptoms or any adverse effects in their hosts [124]. Thus,

this field of research must be further explored as there are still many endophytic microorganisms to be discovered and characterized [124].

3.2. Chemoinformatics Approaches for Obtaining New Fungicides

Metabolomics approaches allow the characterization of the metabolites of an organism at a certain time [147]. It aims to identify low molecular weight chemical compounds in biological systems and, combined with other multi-omics technologies, it can be used to investigate and characterize microbial interactions [148]. The most commonly used techniques for metabolism research and structural elucidation of compounds in microbial metabolomics are nuclear magnetic resonance (NMR) and mass spectrometry (MS), in addition to MS separation techniques, such as gas chromatography–MS (GC-MS), liquid chromatography–MS (LC-MS), and capillary electrophoresis–MS (CE-MS) [148,149].

NMR techniques are fast and simple, can be high-throughput, and require a minimal sample amount. However, the main limitation of these techniques is their low sensitivity (micromolar to nanomolar range) [150,151] and high cost. Alternatively, MS platforms allow high-throughput accurate mass determination and structural elucidation, providing higher sensitivity (femtomolar to attomolar range) when applied together with separation techniques, and can lower the costs, depending on the resolution degree required [148,151]. Due to the vast number of compounds produced and complexity of the metabolism, a single method is unlikely to generate a metabolite profile, but the use of multiple analytical instruments and methods can achieve higher coverage, identify biomarkers, and evaluate drug toxicity, efficacy, and selectivity against various pathogenic fungi [150,152].

Alongside these technologies, computational algorithms were developed to extract data from spectral noise obtained, perform statistical analysis, and identify the pursued compounds. Furthermore, the processed data can be overlaid with metabolic pathways and modeled to predict the outcomes of biological experiments, although they might be computationally demanding [148,149]. These very recent techniques allow the identification of changes in response to stimuli and of novel metabolites with potential antimicrobial activities. Their modulation could lead to a unique tool useful for designing drugs aimed at reducing pesticide usage, while preserving crop productivity [153,154]. When the development of such natural bioactive metabolites falls short for the generation of new antimicrobial agents, other approaches should be applied, such as rational design. In general, rational designs use existing knowledge regarding a molecule's structure or a reaction of interest, combining computational tools and structural knowledge [155]. There are two main chemoinformatics approaches for obtaining new fungicides or other bioactive compounds: structure-based and ligand-based drug design [156]. The first one relies on the knowledge about the three-dimensional structure or the establishment of homology models based on biological receptors, whereas the latter relies on the knowledge about other molecules that bind to the target receptor [156,157].

The high-throughput screening of molecule libraries has advanced drug discovery. This screening relies on quantitative structure–activity relationships (QSAR) and quantitative structure–property relationships (QSPR). Both techniques generate computational models that can predict the biological activity and other properties of a drug based on the molecular structure of a target compound. These models reduce the failure rate of drug targeting, eliminating compounds with previously predicted toxicity or poor pharmacokinetic parameters, optimizing the investigation, and reducing related costs. However, the models are difficult to obtain, and they need to be complemented with the use of other methods, such as molecular modeling, pattern recognition, machine learning or artificial intelligence [158]. In addition, molecular dynamics simulation is also a versatile computational technique to study biological molecules, highly contributing to the development of a rational design at multiple levels. Its combination with other techniques, such as calculation of free energy of molecular docking and binding are essential to elucidate the ligand–receptor interactions, thereby directing a rational investigation [159]. Figure 3 illustrates the schematic process to obtain new drug candidates.

Figure 3. The use of metagenomics in the development of new drug candidates. The left path illustrates metabolite evaluation to identify bioactive or toxic molecules overlaid with metabolic pathways and the selected drug candidate. The right path illustrates the molecular library screening used to generate computational models and calculation of the docking or free energy, leading to the production of a drug candidate.

The use of “omics” techniques and other computational methods, integrated with experimental methods, have allowed the search, prediction, and suggestion of new bioactive molecules and drug candidates, such as agrochemical fungicides, saving time and resources and, thus, becoming an alternative for reducing crop losses and overcoming the problem of antifungal drug resistance [156,160,161].

4. Conclusions

The use of antifungals in agriculture has increased in recent years. Moreover, the recent rate of emergence of fungicide-resistant pathogenic fungi has restricted the number of commonly used antifungal agents. This has led to the need to develop new antifungal agents. However, research to identify and characterize new antifungal drugs is challenging, and the discovery rate of new molecules with antimicrobial potential is less than the emergence rate of new antifungal-resistant strains. Recently,

considerable progress has been made in this field, and the use of nanotechnology has become a promising strategy for the identification of novel antifungal compounds.

Author Contributions: All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding: F.A. acknowledged support from Fundação de Amparo a Pesquisa do Estado de São Paulo (2016/03322-7), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico—420670/2018-1).

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Redecker, D. Glomalean Fungi from the Ordovician. *Science* **2000**, *289*, 1920–1921. [CrossRef] [PubMed]
- Blackwell, M. The Fungi: 1, 2, 3 ... 5.1 million species? *Am. J. Bot.* **2011**, *98*, 426–438. [CrossRef]
- Jampilek, J. Potential of agricultural fungicides for antifungal drug discovery. *Expert. Opin. Drug Discov.* **2016**, *11*, 1–9. [CrossRef] [PubMed]
- Moore, D.; Ahmadjian, V.; Alexopoulos, C.J. Fungus. Encyclopaedia Britannica, 2018. Available online: <https://www.britannica.com/science/fungus/Importance-of-fungi> (accessed on 10 January 2019).
- Köhler, J.R.; Casadevall, A.; Perfect, J. The Spectrum of Fungi That Infects Humans. *Cold Spring Harb. Perspect. Med.* **2014**, *5*, a019273. [CrossRef] [PubMed]
- Mazu, T.K.; Bricker, B.A.; Flores-Rozas, H.; Ablordeppey, S.Y. The Mechanistic Targets of Antifungal Agents: An Overview. *Mini Rev. Med. Chem.* **2016**, *16*, 555–578. [CrossRef] [PubMed]
- Pal, M. Morbidity and Mortality Due to Fungal Infections. *J. Appl. Microbiol. Biochem.* **2017**, *1*, 1–3.
- Maertens, J.; Vrebo, M.; Boogaerts, M. Assessing risk factors for systemic fungal infections. *Eur. J. Cancer Care* **2001**, *10*, 56–62. [CrossRef]
- Almeida, F.; Rodrigues, M.L.; Coelho, C. The Still Underestimated Problem of Fungal Diseases Worldwide. *Front. Microbiol.* **2019**, *10*, 214. [CrossRef] [PubMed]
- Selb, R.; Fuchs, V.; Graf, B.; Hamprecht, A.; Hogardt, M.; Sedlacek, L.; Schwarz, R.; Idelevich, E.A.; Becker, S.L.; Held, J.; et al. Molecular typing and in vitro resistance of *Cryptococcus neoformans* clinical isolates obtained in Germany between 2011 and 2017. *Int. J. Med. Microbiol.* **2019**, *309*, 151336. [CrossRef] [PubMed]
- Brown, G.D.; Denning, D.W.; Gow, N.A.R.; Levitz, S.M.; Netea, M.G.; White, T.C. Hidden Killers: Human Fungal Infections. *Sci. Transl. Med.* **2012**, *4*, 165rv13. [CrossRef]
- Campoy, S.; Adrio, J.L. Antifungals. *Biochem. Pharmacol.* **2017**, *133*, 86–96. [CrossRef] [PubMed]
- Bongomin, F.; Gago, S.; Oladele, R.O.; Denning, D.W. Global and Multi-National Prevalence of Fungal Diseases—Estimate Precision. *J. Fungi* **2017**, *3*, 57. [CrossRef] [PubMed]
- Scorzoni, L.; de Paula e Silva, A.C.; Marcos, C.M.; Assato, P.A.; De Melo, W.C.M.A.; De Oliveira, H.C.; Costa-Orlandi, C.B.; Mendes-Giannini, M.J.S.; Fusco-Almeida, A.M. Antifungal Therapy: New Advances in the Understanding and Treatment of Mycosis. *Front. Microbiol.* **2017**, *8*, 271. [CrossRef] [PubMed]
- Perfect, J.R. The antifungal pipeline: A reality check. *Nat. Rev. Drug Discov.* **2017**, *16*, 603–616. [CrossRef] [PubMed]
- Fisher, M.C.; Hawkins, N.J.; Sanglard, D.; Gurr, S.J. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* **2018**, *360*, 739–742. [CrossRef] [PubMed]
- Perlin, D.S.; Rautemaa-Richardson, R.; Alastruey-Izquierdo, A. The global problem of antifungal resistance: Prevalence, mechanisms, and management. *Lancet Infect. Dis.* **2017**, *17*, e383–e392. [CrossRef]
- Verweij, P.E.; Snelders, E.; Kema, G.H.; Mellado, E.; Melchers, W.J. Azole resistance in *Aspergillus fumigatus*: A side-effect of environmental fungicide use? *Lancet Infect. Dis.* **2009**, *9*, 789–795. [CrossRef]
- Damalas, C.A.; Eleftherohorinos, I.G. Pesticide Exposure, Safety Issues, and Risk Assessment Indicators. *Int. J. Environ. Res. Public Heal.* **2011**, *8*, 1402–1419. [CrossRef]
- Silva, M.F.d.O.e.; Costa, L.M.d. A indústria de defensivos agrícolas. *Agrícolas. BNDES* **2012**, *35*, 233–276.
- Lucas, J.A.; Hawkins, N.J.; Fraaije, B.A. The Evolution of Fungicide Resistance. *Adv. Virus Res.* **2015**, *90*, 29–92.
- James, C. Global Status of Commercialized Biotech/GM Crops: 2010. The International Service for the Acquisition of Agri-biotech Applications (ISAAA): Ithaca, NY, USA, 2010.

23. FAOSTAT. Food and Agriculture Organization of the United Nations. Available online: <http://faostat.fao.org/> (accessed on 17 December 2018).
24. Shuping, D.S.S.; Eloff, J.N. The Use of Plants to Protect Plants and Food Against Fungal Pathogens: A Review. *Afr. J. Tradit. Complement. Altern. Med.* **2017**, *14*, 120–127. [[CrossRef](#)] [[PubMed](#)]
25. Doehlemann, G.; Okmen, B.; Zhu, W.; Sharon, A. Plant Pathogenic Fungi. *Microbiol. Spectr.* **2017**, *5*.
26. Hof, H. Critical Annotations to the Use of Azole Antifungals for Plant Protection. *Antimicrob. Agents Chemother.* **2001**, *45*, 2987–2990. [[CrossRef](#)] [[PubMed](#)]
27. Ribas, A.D.R.E.; Spolti, P.; Del Ponte, E.M.; Donato, K.Z.; Schrekker, H.; Fuentefria, A.M. Is the emergence of fungal resistance to medical triazoles related to their use in the agroecosystems? A mini review. *Braz. J. Microbiol.* **2016**, *47*, 793–799. [[CrossRef](#)]
28. Dean, R.; Van Kan, J.A.L.; Pretorius, Z.A.; Hammond-Kosack, K.E.; Di Pietro, A.; Spanu, P.D.; Rudd, J.J.; Dickman, M.; Kahmann, R.; Ellis, J.; et al. The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 414–430. [[CrossRef](#)] [[PubMed](#)]
29. Villa-Rojas, R.; Sosa-Morales, M.; López-Malo, A.; Tang, J. Thermal inactivation of Botrytis cinerea conidia in synthetic medium and strawberry puree. *Int. J. Food Microbiol.* **2012**, *155*, 269–272. [[CrossRef](#)]
30. Singh, R.P.; Singh, P.K.; Rutkoski, J.; Hodson, D.P.; He, X.; Jørgensen, L.N.; Hovmøller, M.S.; Huerta-Espino, J.; Jørgensen, L.N. Disease Impact on Wheat Yield Potential and Prospects of Genetic Control. *Annu. Rev. Phytopathol.* **2016**, *54*, 303–322. [[CrossRef](#)]
31. Joshi, R.J. A Review on Colletotrichum spp. Virulence mechanism against host plant defensive factors. *J. Med. Plants Stud.* **2018**, *6*, 64–67. [[CrossRef](#)]
32. Griffiths, S.A.; Cox, R.J.; Overdijk, E.J.R.; Mesarich, C.H.; Saccomanno, B.; Lazarus, C.M.; de Wit, P.J.G.M.; Collemare, J. Assignment of a dubious gene cluster to melanin biosynthesis in the tomato fungal pathogen Cladosporium fulvum. *PLoS ONE* **2019**, *13*, e0209600. [[CrossRef](#)]
33. Wang, Y.-Y.; Yin, Q.-S.; Qu, Y.; Li, G.-Z.; Hao, L. Arbuscular mycorrhiza-mediated resistance in tomato against Cladosporium fulvum-induced mould disease. *J. Phytopathol.* **2018**, *166*, 67–74. [[CrossRef](#)]
34. Nikitin, M.; Deych, K.; Grevtseva, I.; Girsova, N.; Kuznetsova, M.; Pridannikov, M.; Dzhavakhiya, V.; Statsyuk, N.; Golikov, A. Preserved Microarrays for Simultaneous Detection and Identification of Six Fungal Potato Pathogens with the Use of Real-Time PCR in Matrix Format. *Biosensors* **2018**, *8*, 129. [[CrossRef](#)] [[PubMed](#)]
35. Saremi, H. Fusarium diseases as the main soil borne fungal pathogen on plants and their control management with soil solarization in Iran. *Afr. J. Biotechnol.* **2011**, *10*, 18391–18398. [[CrossRef](#)]
36. Godfray, H.C.J.; Mason-D'Croz, D.; Robinson, S. Food system consequences of a fungal disease epidemic in a major crop. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2016**, *371*, 20150467. [[CrossRef](#)] [[PubMed](#)]
37. Zhong, Y.; Yan, M.; Jiang, J.; Zhang, Z.; Huang, J.; Zhang, L.; Deng, Y.; Zhou, X.; He, F. Mycophenolic Acid as a Promising Fungal Dimorphism Inhibitor to Control Sugar Cane Disease Caused by Sporisorium scitamineum. *J. Agric. Food Chem.* **2019**, *67*, 112–119. [[CrossRef](#)] [[PubMed](#)]
38. Barnabas, L.; Ashwin, N.M.R.; Kaverinathan, K.; Trentin, A.R.; Pivato, M.; Sundar, A.R.; Malathi, P.; Viswanathan, R.; Rosana, O.O.B.; Neethukrishna, K.; et al. Proteomic analysis of a compatible interaction between sugarcane and Sporisorium scitamineum. *Proteomic* **2016**, *16*, 1111–1122. [[CrossRef](#)] [[PubMed](#)]
39. Large, E.C. Control of Potato Blight (Phytophthora infestans) by Spraying with Suspensions of Metallic Copper. *Nature* **1943**, *151*, 80–81. [[CrossRef](#)]
40. Klittich, C.J. Milestones in Fungicide Discovery: Chemistry that Changed Agriculture. *Plant Heal. Prog.* **2008**, *9*, 31. [[CrossRef](#)]
41. Grassini, P.; Eskridge, K.M.; Cassman, K.G. Distinguishing between yield advances and yield plateaus in historical crop production trends. *Nat. Commun.* **2013**, *4*, 2918. [[CrossRef](#)] [[PubMed](#)]
42. Deising, H.B.; Reimann, S.; Pascholati, S.F. Mechanisms and significance of fungicide resistance. *Braz. J. Microbiol.* **2008**, *39*, 286–295. [[CrossRef](#)]
43. SINDIVEG. National Union of the Product Industry for Plant Protection. Available online: <http://www.sindiveg.org.br/> (accessed on 17 October 2018).
44. Munger, R.; Isacson, P.; Hu, S.; Burns, T.; Hanson, J.; Lynch, C.F.; Cherryholmes, K.; Van Dorpe, P.; Hausler, W.J. Intrauterine growth retardation in Iowa communities with herbicide-contaminated drinking water supplies. *Environ. Heal. Perspect.* **1997**, *105*, 308–314. [[CrossRef](#)]

45. Igbedioh, S.O. Effects of Agricultural Pesticides on Humans, Animals, and Higher Plants in Developing Countries. *Arch. Environ. Heal. Int. J.* **1991**, *46*, 218–224. [[CrossRef](#)] [[PubMed](#)]
46. Lari, S.Z.; Khan, N.A.; Gandhi, K.N.; Meshram, T.S.; Thacker, N.P. Comparison of pesticide residues in surface water and ground water of agriculture intensive areas. *J. Environ. Heal. Sci. Eng.* **2014**, *12*, 11. [[CrossRef](#)] [[PubMed](#)]
47. Vincelli, P. Some Principles of Fungal Resistance. *Plant Pathology Extension*. 2014, pp. 1–10. Available online: <https://plantpathology.ca.uky.edu/files/ppfs-misc-02.pdf> (accessed on 10 January 2019).
48. Cowen, L.E. The evolution of fungal drug resistance: Modulating the trajectory from genotype to phenotype. *Nat. Rev. Genet.* **2008**, *6*, 187–198. [[CrossRef](#)] [[PubMed](#)]
49. Srinivasan, A.; Lopez-Ribot, J.L.; Ramasubramanian, A.K. Overcoming antifungal resistance. *Drug Discov. Today Technol.* **2014**, *11*, 65–71. [[CrossRef](#)] [[PubMed](#)]
50. Brent, K.J.; Hollomon, D.W.; Shaw, M.W. Predicting the Evolution of Fungicide Resistance. *ACS Symposium Series* **1990**, *421*, 303–319.
51. Brent, K.J.; Hollomon, D.W. *Fungicide Resistance in Crop Pathogens: How Can It Be Managed?* The Fungicide Resistance Action Committee: Brussels, Belgium, 2007.
52. Sanglard, D. Emerging Threats in Antifungal-Resistant Fungal Pathogens. *Front. Med.* **2016**, *3*, 165. [[CrossRef](#)] [[PubMed](#)]
53. Davidse, L.C. Benzimidazole Fungicides: Mechanism of Action and Biological Impact. *Annu. Rev. Phytopathol.* **1986**, *24*, 43–65. [[CrossRef](#)]
54. Gupta, P.K.; Aggarwal, M. CHAPTER 52—Toxicity of fungicides. In *Veterinary Toxicology*; Gupta, R.C., Ed.; Academic Press: Oxford, UK, 2007; pp. 587–601.
55. Leroux, P.; Chapeland, F.; Desbrosses, D.; Gredt, M. Patterns of cross-resistance to fungicides in *Botrytis fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. *Crop. Prot.* **1999**, *18*, 687–697. [[CrossRef](#)]
56. Koenraadt, H. Characterization of Mutations in the Beta-Tubulin Gene of Benomyl-Resistant Field Strains of *Venturia inaequalis* and Other Plant Pathogenic Fungi. *Phytopathology* **1992**, *82*, 1348. [[CrossRef](#)]
57. Ma, Z.; Michailides, T.J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop. Prot.* **2005**, *24*, 853–863. [[CrossRef](#)]
58. Avenot, H.F.; Michailides, T.J. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop. Prot.* **2010**, *29*, 643–651. [[CrossRef](#)]
59. Sierotzki, H.; Scalliet, G. A Review of Current Knowledge of Resistance Aspects for the Next-Generation Succinate Dehydrogenase Inhibitor Fungicides. *Phytopathology* **2013**, *103*, 880–887. [[CrossRef](#)] [[PubMed](#)]
60. Bardas, G.A.; Veloukas, T.; Koutita, O.; Karaoglanidis, G.S. Multiple resistance of *Botrytis cinerea* from kiwifruit to SDHIs, QoIs and fungicides of other chemical groups. *Pest Manag. Sci.* **2010**, *66*, 967–973. [[CrossRef](#)] [[PubMed](#)]
61. Avenot, H.F.; Michailides, T.J. Resistance to Boscalid Fungicide in *Alternaria alternata* Isolates from Pistachio in California. *Plant Dis.* **2007**, *91*, 1345–1350. [[CrossRef](#)] [[PubMed](#)]
62. McKay, A.H.; Hagerty, G.C.; Follas, G.B.; Moore, M.S.; Christie, M.S.; Beresford, R.M. Succinate dehydrogenase inhibitor (SDHI) fungicide resistance prevention strategy. *New Zealand Plant Prot.* **2011**, *64*, 119–124.
63. Miyamoto, T.; Ishii, H.; Seko, T.; Kobori, S.; Tomita, Y. Occurrence of *Corynespora cassicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. *Plant Pathol.* **2009**, *58*, 1144–1151. [[CrossRef](#)]
64. Chapeland, F.; Fritz, R.; Lanen, C.; Gredt, M.; Leroux, P. Inheritance and Mechanisms of Resistance to Anilinopyrimidine Fungicides in *Botrytis Cinerea* (*Botrytis fuckeliana*). *Pestic. Biochem. Physiol.* **1999**, *64*, 85–100. [[CrossRef](#)]
65. FRAC. Fungicide Resistance Action Committee. Available online: <http://www.frac.info/home> (accessed on 10 January 2019).
66. Latorre, B.; Spadaro, I.; Rioja, M. Occurrence of resistant strains of *Botrytis cinerea* to anilinopyrimidine fungicides in table grapes in Chile. *Crop. Prot.* **2002**, *21*, 957–961. [[CrossRef](#)]
67. Fiaccadori, R.; Cicognani, E.; Collina, M.; Brunelli, A. Study on the sensitivity of *Venturia inaequalis* to anilinopyrimidine fungicides in Italy. *Commun. Agric. Appl. Biol. Sci.* **2007**, *72*, 997–1001.
68. Mosbach, A.; Edel, D.; Farmer, A.D.; Widdison, S.; Barchietto, T.; Dietrich, R.A.; Corran, A.; Scalliet, G. Anilinopyrimidine Resistance in *Botrytis cinerea* Is Linked to Mitochondrial Function. *Front. Microbiol.* **2017**, *8*, 2361. [[CrossRef](#)]

69. Leroux, P.; Albertini, C.; Lanen, C.; Bach, J.; Gredt, M.; Chapeland, F.; Chapeland-Leclerc, F. Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Manag. Sci.* **2002**, *58*, 876–888. [[CrossRef](#)] [[PubMed](#)]
70. Fernández-Ortuño, D.; Torés, J.A.; De Vicente, A.; Pérez-García, A. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. *Int. Microbiol.* **2008**, *11*, 1–9. [[PubMed](#)]
71. Price, C.L.; Parker, J.E.; Warrilow, A.G.; Kelly, D.E.; Kelly, S.L. Azole fungicides—Understanding resistance mechanisms in agricultural fungal pathogens. *Pest Manag. Sci.* **2015**, *71*, 1054–1058. [[CrossRef](#)]
72. Cools, H.J.; Hawkins, N.; Fraaije, B.A. Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathol.* **2013**, *62*, 36–42. [[CrossRef](#)]
73. Salahuddin, Y.; M.S.; Mazumder, A. Benzimidazoles: A biologically active compounds. *Arab. J. Chem.* **2017**, *10*, S157–S173. [[CrossRef](#)]
74. Singh, N.; Pandurangan, A.; Rana, K.; Anand, P.; Ahamad, A.; Tiwari, A.K. Benzimidazole: A short review of their antimicrobial activities. *Int. Curr. Pharm. J.* **2012**, *1*, 110–118. [[CrossRef](#)]
75. Vela-Corcía, D.; Romero, D.; de Vicente, A.; Pérez-García, A. Analysis of β -tubulin-carbendazim interaction reveals that binding site for MBC fungicides does not include residues involved in fungicide resistance. *Sci. Rep.* **2018**, *8*, 7161. [[CrossRef](#)] [[PubMed](#)]
76. Ermler, S.; Scholze, M.; Kortenkamp, A. Seven benzimidazole pesticides combined at sub-threshold levels induce micronuclei in vitro. *Mutagenesis* **2013**, *28*, 417–426. [[CrossRef](#)] [[PubMed](#)]
77. Hawkins, N.J.; Fraaije, B.A. Predicting Resistance by Mutagenesis: Lessons from 45 Years of MBC Resistance. *Front. Microbiol.* **2016**, *7*, 17. [[CrossRef](#)] [[PubMed](#)]
78. Bartlett, D.W.; Clough, J.M.; Godwin, J.R.; Hall, A.A.; Hamer, M.; Parr-Dobrzanski, B. The strobilurin fungicides. *Pest Manag. Sci.* **2002**, *58*, 649–662. [[CrossRef](#)] [[PubMed](#)]
79. Balba, H. Review of strobilurin fungicide chemicals. *J. Environ. Sci. Heal. Part B* **2007**, *42*, 441–451. [[CrossRef](#)] [[PubMed](#)]
80. Fernández-Ortuño, D.; Torés, J.A.; Antonio, D.; Vicente, D.; Pérez-García, A. *The QoI Fungicides, the Rise and Fall of a Successful Class of Agricultural Fungicides*; Odile Carisse: Rijeka, Croatia, 2010.
81. Grasso, V.; Palermo, S.; Sierotzki, H.; Garibaldi, A.; Gisi, U. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. *Pest Manag. Sci.* **2006**, *62*, 465–472. [[CrossRef](#)] [[PubMed](#)]
82. Ishii, H. Impact of Fungicide Resistance in Plant Pathogens on Crop Disease Control and Agricultural Environment. *Jpn. Agric. Res. Q. JARQ* **2006**, *40*, 205–211. [[CrossRef](#)]
83. Gisi, U.; Sierotzki, H.; Cook, A.; McCaffery, A. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Manag. Sci.* **2002**, *58*, 859–867. [[CrossRef](#)] [[PubMed](#)]
84. Naim, M.J.; Alam, O.; Alam, M.J.; Alam, P.; Shrivastava, N. A review on pharmacological profile of morpholine derivatives. *Int. J. Pharmacol. Pharm. Sci.* **2015**, *3*, 40–51.
85. Mercer, E.I. Morpholine antifungals and their mode of action. *Biochem. Soc. Trans.* **1991**, *19*, 788–793. [[CrossRef](#)]
86. Lago, M.; Aguiar, A.; Natário, A.; Fernandes, C.; Faria, M.; Pinto, E. Does fungicide application in vineyards induce resistance to medical azoles in *Aspergillus* species? *Environ. Monit. Assess.* **2014**, *186*, 5581–5593. [[CrossRef](#)]
87. Azevedo, M.-M.; Faria-Ramos, I.; Cruz, L.C.; Pina-Vaz, C.; Rodrigues, A.G. Genesis of Azole Antifungal Resistance from Agriculture to Clinical Settings. *J. Agric. Food Chem.* **2015**, *63*, 7463–7468. [[CrossRef](#)]
88. Revie, N.M.; Iyer, K.R.; Robbins, N.; Cowen, L.E. Antifungal drug resistance: Evolution, mechanisms and impact. *Curr. Opin. Microbiol.* **2018**, *45*, 70–76. [[CrossRef](#)]
89. Hollomon, D. Does agricultural use of azole fungicides contribute to resistance in the human pathogen *Aspergillus fumigatus*? *Pest Manag. Sci.* **2017**, *73*, 1987–1993. [[CrossRef](#)]
90. Enserink, M. Infectious diseases. Farm fungicides linked to resistance in a human pathogen. *Science* **2009**, *326*, 1173. [[CrossRef](#)] [[PubMed](#)]
91. Kwon-Chung, K.J.; Sugui, J.A. *Aspergillus fumigatus*—What Makes the Species a Ubiquitous Human Fungal Pathogen? *PLoS Pathog.* **2013**, *9*, e1003743. [[CrossRef](#)] [[PubMed](#)]
92. Misch, E.A.; Safdar, N. Updated guidelines for the diagnosis and management of aspergillosis. *J. Thorac. Dis.* **2016**, *8*, E1771–E1776. [[CrossRef](#)] [[PubMed](#)]

93. Berger, S.; El Chazli, Y.; Babu, A.F.; Coste, A.T. Azole Resistance in *Aspergillus fumigatus*: A Consequence of Antifungal Use in Agriculture? *Front. Microbiol.* **2017**, *8*, 1–6. [[CrossRef](#)] [[PubMed](#)]
94. Snelders, E.; van der Lee, H.A.; Kuijpers, J.; Rijs, A.J.; Varga, J.; Samson, R.A.; Mellado, E.; Donders, A.R.; Melchers, W.J.; Verweij, P.E. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. *PLoS Med.* **2008**, *5*, e219. [[CrossRef](#)] [[PubMed](#)]
95. Rivero-Menendez, O.; Alastruey-Izquierdo, A.; Mellado, E.; Cuenca-Estrella, M. Triazole Resistance in *Aspergillus* spp.: A Worldwide Problem? *J. Fungi* **2016**, *2*, 21. [[CrossRef](#)]
96. Hagiwara, D.; Watanabe, A.; Kamei, K.; Goldman, G.H. Epidemiological and Genomic Landscape of Azole Resistance Mechanisms in. *Front. Microbiol.* **2016**, *7*, 1382. [[CrossRef](#)]
97. Bustamante, B.; Illescas, L.R.; Posadas, A.; Campos, P.E. Azole resistance among clinical isolates of *Aspergillus fumigatus* in Lima-Peru. *Med. Mycol.* **2019**. [[CrossRef](#)]
98. Verweij, P.E.; Chowdhary, A.; Melchers, W.J.; Meis, J.F. Azole Resistance in *Aspergillus fumigatus*: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? *Clin. Infect. Dis.* **2016**, *62*, 362–368. [[CrossRef](#)]
99. Van der Linden, J.W.; Snelders, E.; Kampinga, G.A.; Rijnders, B.J.; Mattsson, E.; Debets-Ossenkopp, Y.J.; Kuijper, E.J.; Van Tiel, F.H.; Melchers, W.J.; Verweij, P.E. Clinical implications of azole resistance in *Aspergillus fumigatus*, The Netherlands, 2007–2009. *Emerg. Infect. Dis.* **2011**, *17*, 1846–1854. [[CrossRef](#)]
100. Meireles, L.M.; de Araujo, M.L.; Endringer, D.C.; Fronza, M.; Scherer, R. Change in the clinical antifungal sensitivity profile of *Aspergillus flavus* induced by azole and a benzimidazole fungicide exposure. *Diagn. Microbiol. Infect. Dis.* **2019**, *95*, 171–178. [[CrossRef](#)] [[PubMed](#)]
101. Dos Reis, T.F.; Silva, L.P.; de Castro, P.A.; do Carmo, R.A.; Marini, M.M.; da Silveira, J.F.; Ferreira, B.H.; Rodrigues, F.; Lind, A.L.; Rokas, A.; et al. The *Aspergillus fumigatus* Mismatch Repair MSH2 Homolog Is Important for Virulence and Azole Resistance. *mSphere* **2019**, *4*. [[CrossRef](#)] [[PubMed](#)]
102. Latgé, J.-P. *Aspergillus fumigatus* and Aspergillosis. *Clin. Microbiol. Rev.* **1999**, *12*, 310–350. [[CrossRef](#)] [[PubMed](#)]
103. OERKE, E.-C. Crop losses to pests. *J. Agric. Sci.* **2006**, *144*, 31–43. [[CrossRef](#)]
104. Hahn, M. The rising threat of fungicide resistance in plant pathogenic fungi: Botrytis as a case study. *J. Chem. Boil.* **2014**, *7*, 133–141. [[CrossRef](#)] [[PubMed](#)]
105. Nagpure, A.; Choudhary, B.; Gupta, R.K. Chitinases: In agriculture and human healthcare. *Crit. Rev. Biotechnol.* **2014**, *34*, 215–232. [[CrossRef](#)] [[PubMed](#)]
106. Kanhed, P.; Birla, S.; Gaikwad, S.; Gade, A.; Seabra, A.B.; Rubilar, O.; Duran, N.; Rai, M. In vitro antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. *Mater. Lett.* **2013**, *115*, 13–17. [[CrossRef](#)]
107. Faúndez, G.; Troncoso, M.; Navarrete, P.; Figueroa, G. Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni*. *BMC Microbiol.* **2004**, *4*, 19. [[CrossRef](#)] [[PubMed](#)]
108. Varshney, R.; Bhadauria, S.; Gaur, M.S.; Pasricha, R. Characterization of copper nanoparticles synthesized by a novel microbiological method. *JOM* **2010**, *62*, 102–104. [[CrossRef](#)]
109. Borkow, G.; Gabbay, J. Copper as a biocidal tool. *Curr. Med. Chem.* **2005**, *12*, 2163–2175. [[CrossRef](#)]
110. Lamichhane, J.R.; Osdaghi, E.; Behlau, F.; Kohl, J.; Jones, J.B.; Aubertot, J.-N. Thirteen decades of antimicrobial copper compounds applied in agriculture. A review. *Agron. Sustain. Dev.* **2018**, *38*, 28. [[CrossRef](#)]
111. Sutradhar, P.; Saha, M.; Maiti, D. Microwave synthesis of copper oxide nanoparticles using tea leaf and coffee powder extracts and its antibacterial activity. *J. Nanostructure Chem.* **2014**, *4*, 86. [[CrossRef](#)]
112. Naika, H.R.; Lingaraju, K.; Manjunath, K.; Kumar, D.; Nagaraju, G.; Suresh, D.; Nagabhushana, H. Green synthesis of CuO nanoparticles using *Gloriosa superba* L. extract and their antibacterial activity. *J. Taibah Univ. Sci.* **2015**, *9*, 7–12. [[CrossRef](#)]
113. Duhan, J.S.; Kumar, R.; Kumar, N.; Kaur, P.; Nehra, K.; Duhan, S. Nanotechnology: The new perspective in precision agriculture. *Biotechnol. Rep.* **2017**, *15*, 11–23. [[CrossRef](#)] [[PubMed](#)]
114. Hao, Y.; Cao, X.; Ma, C.; Zhang, Z.; Zhao, N.; Ali, A.; Hou, T.; Xiang, Z.; Zhuang, J.; Wu, S.; et al. Potential Applications and Antifungal Activities of Engineered Nanomaterials against Gray Mold Disease Agent *Botrytis cinerea* on Rose Petals. *Front. Plant Sci.* **2017**, *8*, 1332. [[CrossRef](#)] [[PubMed](#)]
115. Servin, A.; Elmer, W.; Mukherjee, A.; De La Torre-Roche, R.; Hamdi, H.; White, J.C.; Bindraban, P.; Dimkpa, C. A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. *J. Nanoparticle Res.* **2015**, *17*, 92. [[CrossRef](#)]

116. Ditta, A.; Arshad, M. Applications and perspectives of using nanomaterials for sustainable plant nutrition. *Nanotechnol. Rev.* **2016**, *5*, 209. [[CrossRef](#)]
117. Altintas, A.; Tabanca, N.; Tyihak, E.; Ott, P.G.; Moricz, A.M.; Mincsovcics, E.; Wedge, D.E. Characterization of volatile constituents from *Origanum onites* and their antifungal and antibacterial activity. *J. AOAC Int.* **2013**, *96*, 1200–1208. [[CrossRef](#)]
118. Jurick, W.M., 2nd; Macarasin, O.; Gaskins, V.L.; Park, E.; Yu, J.; Janisiewicz, W.; Peter, K.A. Characterization of Postharvest Fungicide-Resistant *Botrytis cinerea* Isolates from Commercially Stored Apple Fruit. *Phytopathology* **2017**, *107*, 362–368. [[CrossRef](#)]
119. Williamson, B.; Tudzynski, B.; Tudzynski, P.; Van Kan, J.A.L. *Botrytis cinerea*: The cause of grey mould disease. *Mol. Plant Pathol.* **2007**, *8*, 561–580. [[CrossRef](#)]
120. Molefi, M.; Chofle, A.A.; Molloy, S.F.; Kalluvya, S.; Chungalucha, J.M.; Cainelli, F.; Leeme, T.; Lekwape, N.; Goldberg, D.W.; Haverkamp, M.; et al. AMBITION-cm: Intermittent high dose AmBisome on a high dose fluconazole backbone for cryptococcal meningitis induction therapy in sub-Saharan Africa: Study protocol for a randomized controlled trial. *Trials* **2015**, *16*, 525. [[CrossRef](#)] [[PubMed](#)]
121. Dooley, H.; Shaw, M.W.; Spink, J.; Kildea, S.; Shaw, M. Effect of azole fungicide mixtures, alternations and dose on azole sensitivity in the wheat pathogen *Zymoseptoria tritici*. *Plant Pathol.* **2015**, *65*, 124–136. [[CrossRef](#)]
122. Ratajczak, M.Z.; Ratajczak, J. Horizontal transfer of RNA and proteins between cells by extracellular microvesicles: 14 years later. *Clin. Transl. Med.* **2016**, *5*, 1487. [[CrossRef](#)] [[PubMed](#)]
123. Wang, M.; Weiberg, A.; Lin, F.-M.; Thomma, B.P.H.J.; Huang, H.-D.; Jin, H. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* **2016**, *2*, 16151. [[CrossRef](#)] [[PubMed](#)]
124. Card, S.; Johnson, L.; Teasdale, S.; Caradus, J. Deciphering endophyte behaviour: The link between endophyte biology and efficacious biological control agents. *FEMS Microbiol. Ecol.* **2016**, *92*, 114. [[CrossRef](#)] [[PubMed](#)]
125. Backman, P.A.; Sikora, R.A. Endophytes: An emerging tool for biological control. *Boil. Control.* **2008**, *46*, 1–3. [[CrossRef](#)]
126. Müller, D.B.; Vogel, C.; Bai, Y.; Vorholt, J.A. The Plant Microbiota: Systems-Level Insights and Perspectives. *Annu. Rev. Genet.* **2016**, *50*, 211–234. [[CrossRef](#)]
127. Deshmukh, S.K.; Gupta, M.K.; Prakash, V.; Saxena, S. Endophytic Fungi: A Source of Potential Antifungal Compounds. *J. Fungi* **2018**, *4*, 77. [[CrossRef](#)]
128. Cai, R.; Jiang, H.; Zang, Z.; Li, C.; She, Z. New Benzofuranoids and Phenylpropanoids from the Mangrove Endophytic Fungus, *Aspergillus* sp. ZJ-68. *Mar. Drugs* **2019**, *17*, 478. [[CrossRef](#)]
129. Xu, Z.; Xiong, B.; Xu, J. Chemical Investigation of Secondary Metabolites Produced By Mangrove Endophytic Fungus *Phyllosticta Capitalensis*. *Nat. Prod. Res.* **2019**, 1–5. [[CrossRef](#)]
130. Chauhan, N.M.; Gutama, A.D.; Aysa, A. Endophytic fungal diversity isolated from different agro-ecosystem of Enset (*Ensete ventricosum*) in Gedeo zone, SNNPRS, Ethiopia. *BMC Microbiol.* **2019**, *19*, 172. [[CrossRef](#)] [[PubMed](#)]
131. Mafezoli, J.; Xu, Y.M.; Hilario, F.; Freidhof, B.; Espinosa-Artiles, P.; Dos Santos, L.C.; de Oliveira, M.C.F.; Gunatilaka, A.A.L. Modulation of polyketide biosynthetic pathway of the endophytic fungus, *Anteaglonium* sp. FL0768, by copper (II) and anacardic acid. *Phytochem. Lett.* **2018**, *28*, 157–163. [[CrossRef](#)] [[PubMed](#)]
132. Kauppinen, M.; Saikkonen, K.; Helander, M.; Pirttilä, A.M.; Wäli, P.R. *Epichloë* grass endophytes in sustainable agriculture. *Nat. Plants* **2016**, *2*, 15224. [[CrossRef](#)] [[PubMed](#)]
133. Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M. *Trichoderma* species—Opportunistic, avirulent plant symbionts. *Nat. Rev. Genet.* **2004**, *2*, 43–56. [[CrossRef](#)] [[PubMed](#)]
134. Rajesh, R.W.; Rahul, M.S.; Ambalal, N.S.; Waghund, R.R.; Shelake, R.M.; Sabalpara, A.N. *Trichoderma*: A significant fungus for agriculture and environment. *Afr. J. Agric. Res.* **2016**, *11*, 1952–1965. [[CrossRef](#)]
135. Woo, S.L.; Ruocco, M.; Vinale, F.; Nigro, M.; Marra, R.; Lombardi, N.; Pascale, A.; Lanzuise, S.; Manganiello, G.; Lorito, M. *Trichoderma*-based Products and their Widespread Use in Agriculture. *Open Mycol. J.* **2014**, *8*, 71–126. [[CrossRef](#)]
136. Almeida, F.B.D.R.; Cerqueira, F.M.; Silva, R.D.N.; Ulhoa, C.J.; Lima, A.L. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: Evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett.* **2007**, *29*, 1189–1193. [[CrossRef](#)]

137. Tang, W.; Yang, H.; Ryder, M. Research and application of *Trichoderma* spp. in biological control of plant pathogens. In *Bio-Exploitation of Filamentous Fungi*; Pointing, S.B., Hyde, K.D., Eds.; Fungal Diversity Press: Hong Kong, China, 2001; pp. 403–435.
138. Nicoletti, R.; Fiorentino, A. Plant Bioactive Metabolites and Drugs Produced by Endophytic Fungi of Spermatophyta. *Agriculture* **2015**, *5*, 918–970. [[CrossRef](#)]
139. Lugtenberg, B.J.; Caradus, J.R.; Johnson, L.J. Fungal endophytes for sustainable crop production. *FEMS Microbiol. Ecol.* **2016**, *92*, 194. [[CrossRef](#)]
140. Morandini, L.M.; Neto, A.T.; Pedroso, M.; Antoniolli, Z.I.; Burrow, R.A.; Bortoluzzi, A.J.; Mostardeiro, M.A.; Da Silva, U.F.; Dalcol, I.I.; Morel, A.F. Lanostane-type triterpenes from the fungal endophyte *Scleroderma* UFSMSc1 (Persoon) Fries. *Bioorganic Med. Chem. Lett.* **2016**, *26*, 1173–1176. [[CrossRef](#)]
141. Macías-Rubalcava, M.L.; Sánchez-Fernández, R.E. Secondary metabolites of endophytic *Xylaria* species with potential applications in medicine and agriculture. *World J. Microbiol. Biotechnol.* **2017**, *33*, 15. [[CrossRef](#)]
142. Wu, S.-H.; He, J.; Li, X.-N.; Huang, R.; Song, F.; Chen, Y.-W.; Miao, C.-P. Guaiane sesquiterpenes and isopimarane diterpenes from an endophytic fungus *Xylaria* sp. *Phytochemistry* **2014**, *105*, 197–204. [[CrossRef](#)]
143. Pereira, C.B.; de Oliveira, D.M.; Hughes, A.F.; Kohlhoff, M.; Vieira, M.L.; Vaz, A.B.M.; Ferreira, M.C.; Carvalho, C.R.; Rosa, L.H.; Rosa, C.A.; et al. Endophytic fungal compounds active against *Cryptococcus neoformans* and *C. gattii*. *J. Antibiot* **2015**, *68*, 436–444. [[CrossRef](#)]
144. Strobel, G.A.; Miller, R.V.; Martinez-Miller, C.; Condrón, M.M.; Teplow, D.B.; Hess, W.M. Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. *Microbiology* **1999**, *145*, 1919–1926. [[CrossRef](#)]
145. Walsh, A.T. Inhibitors of P-glucan synthesis. In *Emerging Targets in Antibacterial and Antifungal Chemotherapy*; Sutcliffe, J.A., Georgopapadakou, N.H., Eds.; Springer: London, UK, 1992; pp. 349–373.
146. Hardoim, P.R.; Van Overbeek, L.S.; Berg, G.; Pirttilä, A.M.; Compant, S.; Campisano, A.; Döring, M.; Sessitsch, A. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiol. Mol. Boil. Rev.* **2015**, *79*, 293–320. [[CrossRef](#)]
147. Joshua, C.J. Metabolomics: A Microbial Physiology and Metabolism Perspective. *Methods Mol. Biol.* **2019**, *1859*, 71–94.
148. Baidoo, E.E.K. Microbial Metabolomics: A General Overview. *Methods Mol. Biol.* **2019**, *1859*, 1–8.
149. Baidoo, E.E.K.; Keasling, J.D. Microbial metabolomics: Welcome to the real world! *Metabolomics* **2013**, *9*, 755–756. [[CrossRef](#)]
150. Johanningsmeier, S.D.; Harris, G.K.; Klevorn, C.M. Metabolomic Technologies for Improving the Quality of Food: Practice and Promise. *Annu. Rev. Food Sci. Technol.* **2016**, *7*, 413–438. [[CrossRef](#)]
151. Caudy, A.A.; Müllender, M.; Ralser, M. Metabolomics in Yeast. *Cold Spring Harb. Protoc.* **2017**, *9*, pdb.top083576. [[CrossRef](#)]
152. Powers, R. NMR metabolomics and drug discovery. *Magn. Reson. Chem.* **2009**, *47*, S2–S11. [[CrossRef](#)]
153. Tian, H.; Lam, S.M.; Shui, G. Metabolomics, a Powerful Tool for Agricultural Research. *Int. J. Mol. Sci.* **2016**, *17*, 1871. [[CrossRef](#)]
154. Arbona, V.; Gómez-Cadenas, A. Metabolomics of Disease Resistance in Crops. *Curr. Issues Mol. Boil.* **2015**, *19*, 13–30.
155. Maeda, Y.; Makhlynets, O.V.; Matsui, H.; Korendovych, I.V. Design of Catalytic Peptides and Proteins Through Rational and Combinatorial Approaches. *Annu. Rev. Biomed. Eng.* **2016**, *18*, 311–328. [[CrossRef](#)]
156. Speck-Planche, A.; Guilarte-Montero, L.; Yera-Bueno, R.; Rojas-Vargas, J.A.; García-López, A.; Molina-Pérez, E.; Uriarte, E. Rational design of new agrochemical fungicides using substructural descriptors. *Pest Manag. Sci.* **2011**, *67*, 438–445. [[CrossRef](#)]
157. Zhang, J.-L.; Zheng, Q.-C.; Chu, W.-T.; Zhang, H.-X. Drug design benefits from molecular dynamics: Some examples. *Curr. Comput. Drug Des.* **2013**, *9*, 532–546. [[CrossRef](#)]
158. Aguiar-Pulido, V.; Gestal, M.; Cruz-Monteagudo, M.; Rabuñal, J.R.; Dorado, J.; Munteanu, C.R. Evolutionary computation and QSAR research. *Curr. Comput. Drug Des.* **2013**, *9*, 206–225. [[CrossRef](#)]
159. Namba, A.M.; Da Silva, V.B.; Da Silva, C.H.T.P. Dinâmica molecular: Teoria e aplicações em planejamento de fármacos. *Eclética Química J.* **2008**, *33*, 13–24. [[CrossRef](#)]

160. Borowski, E. Novel approaches in the rational design of antifungal agents of low toxicity. *Il Farm.* **2000**, *55*, 206–208. [[CrossRef](#)]
161. Guido, R.; Andricopulo, A.D.; Oliva, G. Planejamento de fármacos, biotecnologia e química medicinal: Aplicações em doenças infecciosas. *Estudos Avançados* **2010**, *24*, 81–98. [[CrossRef](#)]

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ANEXO B

Manuscrito: FREITAS, M. S.; BONATO, V. L. D.; PESSONI, A. M.; RODRIGUES, M. L.; CASADEVALL, A.; ALMEIDA, F. Fungal Extracellular Vesicles as Potential Targets for Immune Interventions. mSphere, v.4, p.e00747, 2019.

Fungal Extracellular Vesicles as Potential Targets for Immune Interventions

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ABSTRACT The release of extracellular vesicles (EVs) by fungi is a fundamental cellular process. EVs carry several biomolecules, including pigments, proteins, enzymes, lipids, nucleic acids, and carbohydrates, and are involved in physiological and pathological processes. EVs may play a pivotal role in the establishment of fungal infections, as they can interact with the host immune system to elicit multiple outcomes. It has been observed that, depending on the fungal pathogen, EVs can exacerbate or attenuate fungal infections. The study of the interaction between fungal EVs and the host immune system and understanding of the mechanisms that regulate those interactions might be useful for the development of new adjuvants as well as the improvement of protective immune responses against infectious or noninfectious diseases. In this review, we describe the immunomodulatory properties of EVs produced by pathogenic fungi and discuss their potential as adjuvants for prophylactic or therapeutic strategies.

KEYWORDS drug targets, extracellular vesicles, fungal infections, immune response

Extracellular vesicles (EVs) are spherical structures delimited by a bilayered membrane that are produced by prokaryotic and eukaryotic cells (1, 2). EVs transport various biomolecules, such as proteins, lipids, nucleic acids, and carbohydrates, and they are involved in several aspects of physiology and pathogenicity (2–4). EVs are classified as apoptotic bodies, ectosomes, or exosomes, depending upon their cellular origin and size (5). Apoptotic bodies are the largest (50 to 5,000 nm in size) and are derived from apoptotic cells. Ectosomes, also called microvesicles, are generated by outward budding from the plasma membrane, followed by pinching off and release to the extracellular space, resulting in EVs ranging from 100 to 1,000 nm in size. Exosomes are the smallest EVs (30 to 150 nm), and these structures originate from endosomal compartments (5–7). In this review, we adopt EVs as a general term for all of the above-mentioned vesicles.

In fungi, the first visual evidence of the production of EVs was produced in 1972 in *Aspergillus nidulans* (8). Microscopic evidence of fungal EVs was reproduced in 1973 in *Cryptococcus neoformans* (9), 1990 in *Candida albicans* (10), and 1998 in *Saccharomyces cerevisiae* (11), but the first characterization of extracellular membranous structures as fungal EVs dates to 2007 in the *C. neoformans* model (12). So far, the production of EVs has been observed in a number of fungal species (13–19). The composition of fungal EVs can vary, depending on the availability of nutrition and the immunological activity of host cells, and they typically contain proteins, RNA, lipids, complex carbohydrates, and pigments (20, 21). Due to the heterogeneity in their content, fungal EVs are able

Citation Freitas MS, Bonato VLD, Pessoni AM, Rodrigues ML, Casadevall A, Almeida F. 2019. Fungal extracellular vesicles as potential targets for immune interventions. *mSphere* 4:e00747-19. <https://doi.org/10.1128/mSphere.00747-19>.

Editor Aaron P. Mitchell, Carnegie Mellon University

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Published 6 November 2019

to participate in a number of physiological processes, including biofilm formation, the transport of virulence factors, and modulation of the host immune response (22, 23).

Deep mycoses, such as cryptococcosis, candidiasis, and aspergillosis, are responsible for approximately 1,270,000 annual global cases, and the mortality rates from these mycoses are comparable to those from malaria (24, 25). The drugs currently approved for treating human mycoses usually have low efficacy and high toxicity, and the widespread use of these medications is selecting for resistant strains (26–29). Given the high incidence of fungal diseases worldwide and their therapeutic limitations, it is important to study the biology of pathogenic fungi in an attempt to develop new immune interventions (30). In this review, we discuss the immunomodulatory potential of fungal EVs. Additionally, we highlight strategies where fungal EVs could be used as therapeutic targets and/or as components of therapeutic and prophylactic strategies.

THE INTERACTION OF FUNGAL EVs WITH THE IMMUNE SYSTEM

Most of the data resulting from the immunomodulatory effects of EVs are derived from studies involving Gram-negative bacteria (31). Macrophages that internalize *Neisseria gonorrhoeae* EVs undergo apoptosis due to the presence of the porin PorB within the vesicles, resulting in altered mitochondrial permeability and cytochrome c release (32). EVs derived from *Escherichia coli* can also cause apoptosis in human intestinal epithelial cells due to interleukin-8 (IL-8) production, possibly mediated by the intracellular receptor NOD-1 (33, 34). Similar effects were observed in Gram-positive bacteria, where the listeriolysin O within EVs produced by *Listeria monocytogenes* decreased the viability of J774 macrophages (35). *Streptococcus pneumoniae* EVs also induce inflammatory cytokine production and cell maturation (36). Additionally, EVs derived from *Streptococcus pneumoniae* interact with complement components that cannot directly interact with the bacteria, thus avoiding phagocytosis (36). Fungal EVs also possess immunogenic properties (37). The proteins, RNA, lipids, carbohydrates, and pigments in fungal EVs are recognized by pattern recognition receptors (PRRs) expressed on leukocytes and activate immune responses (38). These collective findings show that EVs of fungi might positively or negatively modulate the activation of innate immunity.

Cryptococcus. *C. neoformans* is the principal causative agent of cryptococcosis, a disease distributed worldwide. After inhalation of fungal cells, immunosuppressed individuals, such as those infected with HIV, can develop the invasive form of this disease (39, 40). EVs derived from *C. neoformans* carry many virulence factors, including its major capsular antigen, glucuronoxylomannan (GXM), and laccase, the enzyme responsible for melanin production (12, 41). GXM exerts an immunosuppressive action over macrophages, monocytes, neutrophils, and T lymphocytes (42). This polysaccharide enhances IL-10 production by monocytes, subsequently impairing IL-12 production and intracellular killing (43). The lack of IL-12 may be due to the low levels of production of gamma interferon (IFN- γ) by peripheral blood mononuclear cells (PBMC), which in turn hampers the development of the Th1 protective response (43). GXM also exerts a direct *in vivo* and *in vitro* cytotoxic effect on macrophages due to activation of the Fas/FasL pathway (44, 45). Indeed, it has been demonstrated that macrophages stimulated *in vitro* with EVs derived from *C. neoformans* produce anti-inflammatory cytokines, such as transforming growth factor β (TGF- β) and IL-10 (46). Interestingly, the production of both TNF- α and nitric oxide (NO), as well as an increased ability to phagocytize and kill fungal cells, suggests that several molecules present in EVs derived from *C. neoformans* play dual roles: positive and negative stimulation of macrophages (46). These findings reinforce the suggestion that the effect of *C. neoformans* EVs is more protective than deleterious for the host. The presence of specific antibodies against EV proteins in the sera of patients diagnosed with cryptococcosis confirms the activation of the humoral immune response by these EVs *in vivo* (41). The degradation of cryptococcal EVs in human sera might be highly efficient, since the vesicles are disrupted in the presence of albumin and galectin-3 (47, 48).

EVs derived from a virulent strain of *C. gattii* also modulate macrophage responses, ultimately facilitating the intracellular multiplication of less virulent strains without interfering with the phagocytosis rate (49). Therefore, the presence of fungal virulence factors might be critical for the protective or deleterious effect of EVs. Heat inactivation of EVs abolished this effect, suggesting that, besides the virulence factors, a plethora of compounds derived from EVs (lipids, RNA, and proteins) could play a pivotal role in the modulation of macrophage functions (49).

Candida. *C. albicans* is the most common human commensal fungus. This fungus primarily colonizes the gastrointestinal tract and the oral and vaginal mucosa of healthy individuals. In immunosuppressed individuals, *C. albicans* can become an opportunistic pathogen, ultimately causing a range of diseases from mucosal infections to candidemia and disseminated candidiasis (50, 51). Collected findings show that *C. albicans* EVs exhibit immunomodulatory effects that benefit the activation of the innate response. When stimulated with EVs, RAW 264.7 macrophages produce NO, IL-12p40, and lower concentrations of IL-10 and TGF- β , while bone marrow-derived macrophages (BMDM) produce NO, IL-12p40, tumor necrosis factor alpha (TNF- α), and IL-10. Bone marrow-derived dendritic cells (BMDC) produce IL-12p40, TNF- α , and TGF- β and undergo cell maturation that coincides with a higher level of expression of major histocompatibility complex class II and CD86. The hallmark of dendritic cell (DC) activation by *C. albicans* EVs suggests that vesicle constituents might be important for the immune adaptive response against the pathogen (52). The phospholipids contained in *C. albicans* EVs may also play a role in immune cell activation, as EVs derived from phosphatidylserine synthase (CHO1)-knockout fungi were unable to activate NF- κ B in BMDM and J774.14 macrophages (53). EVs produced by *C. albicans* may be more stable in human sera than other fungal vesicles due to the presence of the glucose transmembrane transporter Hgt1p, which binds to complement factor H to allow for evasion of complement alternative pathway activation and, ultimately, opsonization (54). Indeed, blockage of Hgt1p in fungal cells results in an increase in neutrophil phagocytosis and intracellular killing (54). The EVs produced by *C. albicans* also played a protective effect in a *Galleria mellonella* model of fungal infection. Inoculation of EVs into the larval form of this invertebrate 2 days prior to lethal challenge with live *C. albicans* fungal cells resulted in a lower fungal burden and an increased life span in the EV-treated larvae (52).

Paracoccidioides. Paracoccidioidomycosis is a fungal infection endemic in Latin America that is caused by the thermally dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*. The infection initiates in the lungs but can spread to other organs (55). The activation of a Th1 immune response is an indicator of a good prognosis, while the activation of the Th2 and Th9 immune responses causes an uncontrolled, deleterious inflammatory process (56). It is well-known that classically activated macrophages (M1) play a protective role against *P. brasiliensis* (55, 57). Interestingly, it has been demonstrated that *in vitro* stimulation with *P. brasiliensis* EVs can induce the differentiation of M1 macrophages (58). The EV-induced stimulus leads to the production of proinflammatory cytokines and to the increased relative expression of the inducible nitric oxide synthase (iNOS) gene. Additionally, macrophage phagocytosis and intracellular killing were comparable to those that were observed after IFN- γ stimulation (58). Mammalian lectin microarray assays demonstrated that the interaction between EVs and DCs was dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) dependent, with no involvement of Dectin-1 and -2 (59). Sera obtained from patients diagnosed with paracoccidioidomycosis reacted with EVs through a process that required fungal α -galactosyl epitopes (15). Despite this, it remains unclear if the α -galactosyl epitopes in EVs participate in the generation of immune responses, as observed in *Trypanosoma cruzi* (15). As previously reported for *C. neoformans*, galectin-3 disrupts *P. brasiliensis* EVs, ultimately limiting the functionality of those vesicles (60).

Histoplasma. *Histoplasma capsulatum* is a thermally dimorphic fungus that causes histoplasmosis. *H. capsulatum* is distributed worldwide and is highly endemic in North

and South America (61). In immunocompetent hosts, a Th1/Th17 immune response limits the progression of the infection (62). However, a severe form of histoplasmosis might occur in immunocompromised individuals (63, 64). Similar to the findings for other fungal EVs, vesicular proteins of *H. capsulatum* were recognized by sera from patients with histoplasmosis, with a dominant serological reactivity of heat shock protein 60 (Hsp60) and histone 2B (13). The *in vivo* generation of anti-Hsp60 antibodies might be of significance, as it has been demonstrated that *in vitro* incubation of EVs with anti-Hsp60 antibodies alters the morphology, protein cargo, and enzymatic activities (65). Although the interplay of *H. capsulatum* EVs with host leukocytes remains to be better investigated, those EVs reduced the phagocytic rates and intracellular fungal killing by BMDM (66), suggesting that they may be more suppressive than protective in the infection.

Malassezia. *Malassezia* spp. are dimorphic fungi that colonize the human skin as commensal organisms and are typically harmless; however, these fungi can cause skin-related disorders (67). *Malassezia sympodialis* and *M. furfur* are two of the many *Malassezia* species associated with atopic eczema (AE) (68). *M. sympodialis* produces EVs that carry allergens recognized by IgE from AE patients, and those EVs might induce the production of IL-4 and TNF- α by peripheral blood mononuclear cells (PBMC) obtained from AE patients or from healthy individuals (14). *M. sympodialis* EVs also exert an indirect immune effect, as DCs are able to phagocytize these fungal structures and produce their own EVs containing *M. sympodialis* antigens. DC-derived vesicles also induce the production of IL-4 and TNF- α by PBMCs from AE patients (14). In addition to DCs, monocytes and keratinocytes could actively internalize *M. sympodialis* EVs (69). *M. furfur* EVs may also play a role in immune modulation and AE development. Collected evidence from *in vitro* and *in vivo* experiments showed that *M. furfur* EVs stimulated NF- κ B activation and increased IL-1 β gene expression and IL-6 production by keratinocytes (70).

Trichophyton. Dermatophytes are associated with infections of keratin-rich tissues that represent the most common superficial mycoses in humans (24, 71). Dermatophyte infections are also called ringworms or tinea. *Trichophyton interdigitale* and *T. rubrum* are established as the most important species that cause skin and nail infections (71). *T. interdigitale* EVs induce a strong inflammatory response in BMDM and keratinocytes. Both types of cells produce NO, TNF- α , IL-6, and IL-1 β in response to EVs. An increase in iNOS relative expression suggests that EV-treated macrophages differentiate into M1 macrophages, which exhibit a higher phagocytic and intracellular killing index than other macrophages when incubated with fungal conidia. Additionally, Toll-like receptor 2 activation was found to be required for EV-induced macrophage activation (18). These data suggest that *T. interdigitale* EVs positively modulate inflammatory and microbicidal innate responses.

Sporothrix. Sporotrichosis, caused by *Sporothrix* spp., is a mycosis endemic in tropical and subtropical areas (72). In Brazil, *Sporothrix brasiliensis* is the main etiological agent of sporotrichosis, but *Sporothrix* spp. may be distributed worldwide (73, 74). After contact with *Sporothrix* species, both immunocompetent and immunosuppressed individuals can develop sporotrichosis, which varies from cutaneous manifestations to colonization of multiple organs (63, 72). EVs derived from *S. brasiliensis* exhibited immunoreactivity with sera obtained from mice with experimental sporotrichosis, revealing a humoral response against vesicle proteins (19). *S. brasiliensis* EVs played no role in cytokine production by BMDC. However, pretreatment of BMDC with EVs and subsequent exposure to *S. brasiliensis* yeast cells resulted in the differential production of IL-12 p40, TNF- α , and IFN- γ compared with that by nontreated BMDC and higher levels of phagocytosis but not intracellular killing of *S. brasiliensis* yeast cells by pretreated BMDC than by nontreated BMDC. The *in vivo* administration of EVs impaired the initial immune response, as initial skin lesions were larger and contained higher levels of fungal colonization (19). These results suggest that *S. brasiliensis* EVs may be more suppressive than protective in the infection.

FUNGAL EVs AS TARGETS FOR IMMUNE INTERVENTIONS

The urgent need to develop new strategies to prevent and combat fungal infections is efficiently illustrated by the global number of deaths (1.5 million annually) caused by invasive mycoses. There are no licensed antifungal vaccines, and only two vaccine candidates targeting vulvovaginal candidiasis are currently under development (75, 76).

Several groups have demonstrated that fungal EVs act as immunomodulators, as they carry a number of immunogenic molecules that elicit inflammatory and microbicidal responses, suggesting the possibility of their use as candidates for adjuvants, vaccines against invasive fungal infections, or immunotherapies. In this sense, liposomes resembling EVs could be promising structures for the development of vaccines. Liposomes are laboratory-generated round vesicles of various sizes that are formed by one or more lipid bilayers. They can be engineered to carry selected antigens in association with the lipid bilayer or within the vesicle core. Addition of pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns (DAMP) in liposomes promotes adjuvant-like properties (77, 78). In this context, EVs would play a role of natural liposomes, delivering fungal PAMP for leukocytes of the innate system and, possibly, driving the differentiation of CD4⁺ subsets. P10 is an epitope derived from *P. brasiliensis* glycoprotein 43 (gp43) that exerts a protective effect against experimental paracoccidioidomycosis. gp43, which is present in EVs, is the most well studied antigen from *P. brasiliensis* (79). The immunization of healthy or immunosuppressed mice with P10 decreased *P. brasiliensis* fungal loads and increased the survival of mice lethally challenged with *P. brasiliensis* by a mechanism dependent on the Th1 immune response (80–82). This protection is achieved through the establishment of a Th1 response (80). Additionally, concomitant treatment of mice with P10 and antifungal drugs efficiently diminished the fungal burden in infected mice (81). Two facts—that P10 is a component of EVs and that EVs induce the differentiation of M1 macrophages—suggest that the protective potential of *P. brasiliensis* EVs as a candidate vaccine could be assessed.

The *C. albicans* protein 1,3- β -glucosyltransferase (Bgl2) is involved in cell wall biosynthesis and virulence. Bgl2 proteins are present in *C. albicans* EVs and elicit humoral immune responses, as concluded from their reactivity with sera from patients suffering from systemic candidiasis (83). Additionally, treatment of mice with Bgl2 in a vaccine model led to the increased survival of mice challenged with *C. albicans* (84). *C. albicans* EVs used as immunogens in *G. mellonella* reduced the fungal burden and increased larva survival after challenge with yeast cells (52). In this sense, *G. mellonella* provides a useful experimental model, as its innate immune response resembles the human innate immune response. Unlike humans, however, no adaptive immune response is present in this invertebrate. *G. mellonella* exhibits a short period of immune boost after an immune challenge. This period is called “primary immunization” or “primitive immunity” and is characterized by higher counts of hemocytes and the increased production of antimicrobial peptides (85). Thus, the protective effect of *C. albicans* EVs may be due to primary immunization rather than adaptive immunization.

In *C. neoformans*, fungal protein extracts or the recombinant proteins were able to induce protection in mice challenged with *C. neoformans* or *C. gattii* (86, 87). Similarly, the vaccination of mice with protein extracts derived from the cell wall or cytoplasm of *C. gattii* increased the survival of mice and decreased the fungal burden after challenge with *C. gattii* (88). The recruitment of lymphocytes to the infection sites was also reported (88). The association of these proteins with EVs has not been demonstrated, but on the basis of the complexity of the protein composition of EVs, results similar to those described above for protein extracts from *C. gattii* would not be surprising. Indeed, EVs produced by *C. neoformans* containing GXM and sterol glucosides induced protection in *G. mellonella* (89). Similar to what has been proposed for *C. albicans*, this effect could be related to a primary immunization mechanism.

EVs carry several virulence factors, suggesting that detrimental activity against the host cannot be ruled out. EVs transport pathogen antigens that can interact with the

FIG 1 Fungal EVs may play a pivotal role in the establishment of fungal infections and can alter the infection process. These EVs are potential targets for new antifungal agents, as well as potential candidates for chemotherapy and vaccine development.

host immune system, ultimately resulting in immune memory. The controlled administration of fungal EVs in combination with an appropriate three-dimensional culture or animal model could be exploited to provide an adjuvant, a vaccine platform, or an immunotherapy platform. However, there is an equivocal with two commercially available vaccines derived from extracts of outer membrane vesicles from *Neisseria meningitidis* processed into a liposomal form (Bexsero and VA-Mengoc-BC); although these vaccines exhibit satisfactory efficacy (90–93), these materials cannot be considered EVs since these vaccines do not derive from secretions produced by bacterial cells (94). However, these previous successful approaches support the search for vaccines derived from fungal EVs.

The field of fungal EVs is still in its infancy, which agrees with the existence of many gaps in knowledge on how these structures can impact disease. Dissection of the physiological and pathogenic roles of fungal EVs will enhance our understanding of their potential applicability in models of vaccination. In this sense, we propose strategies to explore fungal EVs as potential prophylactic or therapeutic targets (Fig. 1).

We described above the interactions between various pathogenic fungal EVs and leukocytes of the innate system. The diversity of molecules present within these structures results in a diverse immune response. In some cases, the interactions between EVs and the immune system are beneficial to the host, and in other situations, the vesicles support disease development. It is clear that not all EV interactions are well established. Indeed, fungal EVs are important factors in immune response modulation, and they act at different levels, depending on the pathogen and the EV cargo. More studies are necessary to further elucidate this relationship and the possible use of such vesicles in host protection (23). The complexity of fungal EVs is enormous, but their potential to impact the interaction of fungi with host cells suggests that they have great potential in the development of new strategies to combat fungal infections.

ACKNOWLEDGMENTS

We acknowledge support from the Fundação de Amparo a Pesquisa do Estado de São Paulo (grant 2016/03322-7), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and the Instituto Nacional de Ciência e Tecnologia de Inovação em Doenças de Populações Negligenciadas (INCT-IDPN).

REFERENCES

- Deatherage BL, Cookson BT. 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infect Immun* 80:1948–1957. <https://doi.org/10.1128/IAI.06014-11>.
- Yáñez-Mó M, Siljander PR-M, Andreu Z, Zavec AB, Borràs FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, Colás E, Cordeiro-da Silva A, Fais S, Falcon-Perez JM, Ghobrial IM, Giebel B, Gimona M, Graner M, Gursel I, Gursel M, Heegaard NHH, Hendrix A, Kierulff P, Kokubun K, Kosanovic M, Kralj-Iglic V, Krämer-Albers E-M, Laitinen S, Lässer C, Lener T, Ligeti E, Liné A, Lipps G, Llorente A, Lötvall J, Manček-Keber M, Marcilla A, Mittelbrunn M, Nazarenko I, Nolte-’t Hoen ENM, Nyman TA, O’Driscoll L, Olivan M, Oliveira C, Pällinger É, Del Portillo HA, Reventós J, Rigau M, Rohde E, Sammar M, et al. 2015. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* 4:27066. <https://doi.org/10.3402/jev.v4.27066>.
- van Niel G, D’Angelo G, Raposo G. 2018. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol* 19:213–228. <https://doi.org/10.1038/nrm.2017.125>.
- Brock CK, Wallin ST, Ruiz OE, Samms KM, Mandal A, Sumner EA, Eisenhoffer GT. 2019. Stem cell proliferation is induced by apoptotic bodies from dying cells during epithelial tissue maintenance. *Nat Commun* 10:1044. <https://doi.org/10.1038/s41467-019-09010-6>.
- Mallocci M, Perdomo L, Veerasamy M, Andriantsitohaina R, Simard G, Martinez MC. 2019. Extracellular vesicles: mechanisms in human health and disease. *Antioxid Redox Signal* 30:813–856. <https://doi.org/10.1089/ars.2017.7265>.
- Raposo G, Stoorvogel W. 2013. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 200:373–383. <https://doi.org/10.1083/jcb.201211138>.
- Maas SLN, Breakefield XO, Weaver AM. 2017. Extracellular vesicles: unique intercellular delivery vehicles. *Trends Cell Biol* 27:172–188. <https://doi.org/10.1016/j.tcb.2016.11.003>.
- Gibson RK, Peberdy JF. 1972. Fine structure of protoplasts of *Aspergillus nidulans*. *J Gen Microbiol* 72:529–538. <https://doi.org/10.1099/00221287-72-3-529>.
- Takeo K, Uesaka I, Uehira K, Nishiura M. 1973. Fine structure of *Cryptococcus neoformans* grown in vivo as observed by freeze-etching. *J Bacteriol* 113:1449–1454.
- Anderson J, Mihalik R, Soll DR. 1990. Ultrastructure and antigenicity of the unique cell wall pimple of the *Candida* opaque phenotype. *J Bacteriol* 172:224–235. <https://doi.org/10.1128/jb.172.1.224-235.1990>.
- Osumi M. 1998. The ultrastructure of yeast: cell wall structure and formation. *Micron* 29:207–233. [https://doi.org/10.1016/S0968-4328\(97\)00072-3](https://doi.org/10.1016/S0968-4328(97)00072-3).
- Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O, Alvarez M, Nakouzi A, Feldmesser M, Casadevall A. 2007. Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot Cell* 6:48–59. <https://doi.org/10.1128/EC.00318-06>.
- Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancoppe-Oliveira RM, Almeida IC, Nosanchuk JD. 2008. Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. *Cell Microbiol* 10:1695–1710. <https://doi.org/10.1111/j.1462-5822.2008.01160.x>.
- Gehrmann U, Qazi KR, Johansson C, Hulténby K, Karlsson M, Lundberg L, Gabriellsson S, Scheynius A. 2011. Nanovesicles from *Malassezia sympodialis* and host exosomes induce cytokine responses—novel mechanisms for host-microbe interactions in atopic eczema. *PLoS One* 6:e21480. <https://doi.org/10.1371/journal.pone.0021480>.
- Vallejo MC, Matsuo AL, Ganiko L, Medeiros LC, Miranda K, Silva LS, Freymüller-Haapalainen E, Sinigaglia-Coimbra R, Almeida IC, Puccia R. 2011. The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic alpha-galactosyl epitopes. *Eukaryot Cell* 10:343–351. <https://doi.org/10.1128/EC.00227-10>.
- Silva BMA, Prados-Rosales R, Espadas-Moreno J, Wolf JM, Luque-García JL, Gonçalves T, Casadevall A. 2014. Characterization of *Alternaria* infection extracellular vesicles. *Med Mycol* 52:202–210. <https://doi.org/10.1093/mmy/myt003>.
- Leone F, Bellani L, Muccifora S, Giorgetti L, Bongioanni P, Simili M, Maserti B, Del Carratore R. 2018. Analysis of extracellular vesicles produced in the biofilm by the dimorphic yeast *Pichia fermentans*. *J Cell Physiol* 233:2759–2767. <https://doi.org/10.1002/jcp.25885>.
- Bitencourt TA, Rezende CP, Quaresimin NR, Moreno P, Hatanaka O, Rossi A, Martinez-Rossi NM, Almeida F. 2018. Extracellular vesicles from the dermatophyte *Trichophyton interdigitale* modulate macrophage and keratinocyte functions. *Front Immunol* 9:2343. <https://doi.org/10.3389/fimmu.2018.02343>.
- Ikedá MAK, de Almeida JRF, Jannuzzi GP, Cronemberger-Andrade A, Torrecilhas ACT, Moretti NS, da Cunha JPC, de Almeida SR, Ferreira KS. 2018. Extracellular vesicles from *Sporothrix brasiliensis* are an important virulence factor that induce an increase in fungal burden in experimental sporotrichosis. *Front Microbiol* 9:2286. <https://doi.org/10.3389/fmicb.2018.02286>.
- de Toledo Martins S, Szwarc P, Goldenberg S, Alves LR. 2019. Extracellular vesicles in fungi: composition and functions. *Curr Top Microbiol Immunol* 422:45–59. https://doi.org/10.1007/82_2018_141.
- Bleackley MR, Dawson CS, Anderson MA. 2019. Fungal extracellular vesicles with a focus on proteomic analysis. *Proteomics* 19:e1800232. <https://doi.org/10.1002/pmic.201800232>.
- Rella A, Farnoud AM, Del Poeta M. 2016. Plasma membrane lipids and their role in fungal virulence. *Prog Lipid Res* 61:63–72. <https://doi.org/10.1016/j.plipres.2015.11.003>.
- Zamith-Miranda D, Nimrichter L, Rodrigues ML, Nosanchuk JD. 2018. Fungal extracellular vesicles: modulating host-pathogen interactions by both the fungus and the host. *Microbes Infect* 20:501–504. <https://doi.org/10.1016/j.micinf.2018.01.011>.
- Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. *Sci Transl Med* 4:165rv113. <https://doi.org/10.1126/scitranslmed.3004404>.
- Bongomin F, Gago S, Oladele RO, Denning DW. 2017. Global and multi-national prevalence of fungal diseases—estimate precision. *J Fungi (Basel)* 3:E57. <https://doi.org/10.3390/jof3040057>.
- Farmakiotis D, Kontoyiannis DP. 2017. Epidemiology of antifungal resistance in human pathogenic yeasts: current viewpoint and practical recommendations for management. *Int J Antimicrob Agents* 50:318–324. <https://doi.org/10.1016/j.ijantimicag.2017.05.019>.
- Robbins N, Caplan T, Cowen LE. 2017. Molecular evolution of antifungal drug resistance. *Annu Rev Microbiol* 71:753–775. <https://doi.org/10.1146/annurev-micro-030117-020345>.
- Gao J, Wang H, Li Z, Wong AH, Wang YZ, Guo Y, Lin X, Zeng G, Liu H, Wang Y, Wang J. 2018. *Candida albicans* gains azole resistance by altering sphingolipid composition. *Nat Commun* 9:4495. <https://doi.org/10.1038/s41467-018-06944-1>.
- Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD, Fortwendel JR. 2019. Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus*. *mBio* 10:e00437-19. <https://doi.org/10.1128/mBio.00437-19>.
- Almeida F, Rodrigues ML, Coelho C. 2019. The still underestimated problem of fungal diseases worldwide. *Front Microbiol* 10:214. <https://doi.org/10.3389/fmicb.2019.00214>.
- Kuipers ME, Hokke CH, Smits HH, Nolte-’t Hoen ENM. 2018. Pathogen-derived extracellular vesicle-associated molecules that affect the host immune system: an overview. *Front Microbiol* 9:2182. <https://doi.org/10.3389/fmicb.2018.02182>.

32. Deo P, Chow SH, Hay ID, Kleifeld O, Costin A, Elgass KD, Jiang JH, Ramm G, Gabriel K, Dougan G, Lithgow T, Heinz E, Naderer T. 2018. Outer membrane vesicles from *Neisseria gonorrhoeae* target PorB to mitochondria and induce apoptosis. *PLoS Pathog* 14:e1006945. <https://doi.org/10.1371/journal.ppat.1006945>.
33. Kunsman L, Ruter C, Bauwens A, Greune L, Gluder M, Kemper B, Fruth A, Wai SN, He X, Llobes R, Schmidt MA, Dobrindt U, Mellmann A, Karch H, Bielaszewska M. 2015. Virulence from vesicles: novel mechanisms of host cell injury by *Escherichia coli* O104:H4 outbreak strain. *Sci Rep* 5:13252. <https://doi.org/10.1038/srep13252>.
34. Canas MA, Fabrega MJ, Gimenez R, Badia J, Baldoma L. 2018. Outer membrane vesicles from probiotic and commensal *Escherichia coli* activate NOD1-mediated immune responses in intestinal epithelial cells. *Front Microbiol* 9:498. <https://doi.org/10.3389/fmicb.2018.00498>.
35. Coelho C, Brown L, Maryam M, Vij R, Smith DFQ, Burnet MC, Kyle JE, Heyman HM, Ramirez J, Prados-Rosales R, Lauvau G, Nakayasu ES, Brady NR, Hamacher-Brady A, Coppens I, Casadevall A. 2019. *Listeria monocytogenes* virulence factors, including listeriolysin O, are secreted in biologically active extracellular vesicles. *J Biol Chem* 294:1202–1217. <https://doi.org/10.1074/jbc.RA118.006472>.
36. Codemo M, Muschiol S, Iovino F, Nannapaneni P, Plant L, Wai SN, Henriques-Normark B. 2018. Immunomodulatory effects of pneumococcal extracellular vesicles on cellular and humoral host defenses. *mBio* 9:e00559-18. <https://doi.org/10.1128/mBio.00559-18>.
37. Joffe LS, Nimrichter L, Rodrigues ML, Del Poeta M. 2016. Potential roles of fungal extracellular vesicles during infection. *mSphere* 1:e00099-16. <https://doi.org/10.1128/mSphere.00099-16>.
38. Patin EC, Thompson A, Orr SJ. 2019. Pattern recognition receptors in fungal immunity. *Semin Cell Dev Biol* 89:24–33. <https://doi.org/10.1016/j.semcdb.2018.03.003>.
39. May RC, Stone NR, Wiesner DL, Bicanic T, Nielsen K. 2016. *Cryptococcus*: from environmental saprophyte to global pathogen. *Nat Rev Microbiol* 14:106–117. <https://doi.org/10.1038/nrmicro.2015.6>.
40. Boulware DR. 2011. *Cryptococcus*: from human pathogen to model yeast. *Lancet Infect Dis* 11:434. [https://doi.org/10.1016/S1473-3099\(11\)70140-2](https://doi.org/10.1016/S1473-3099(11)70140-2).
41. Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, Nosanchuk JD, Almeida IC, Casadevall A. 2008. Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryot Cell* 7:58–67. <https://doi.org/10.1128/EC.00370-07>.
42. Monari C, Bistoni F, Vecchiarelli A. 2006. Glucuronoxylomannan exhibits potent immunosuppressive properties. *FEMS Yeast Res* 6:537–542. <https://doi.org/10.1111/j.1567-1364.2006.00072.x>.
43. Retini C, Kozel TR, Pietrella D, Monari C, Bistoni F, Vecchiarelli A. 2001. Interdependency of interleukin-10 and interleukin-12 in regulation of T-cell differentiation and effector function of monocytes in response to stimulation with *Cryptococcus neoformans*. *Infect Immun* 69:6064–6073. <https://doi.org/10.1128/IAI.69.10.6064-6073.2001>.
44. Villena SN, Pinheiro RO, Pinheiro CS, Nunes MP, Takiya CM, DosReis GA, Prevato JO, Mendonca-Prevato L, Freire-de-Lima CG. 2008. Capsular polysaccharides galactoxylomannan and glucuronoxylomannan from *Cryptococcus neoformans* induce macrophage apoptosis mediated by Fas ligand. *Cell Microbiol* 10:1274–1285. <https://doi.org/10.1111/j.1462-5822.2008.01125.x>.
45. Monari C, Pericolini E, Bistoni G, Casadevall A, Kozel TR, Vecchiarelli A. 2005. *Cryptococcus neoformans* capsular glucuronoxylomannan induces expression of Fas ligand in macrophages. *J Immunol* 174:3461–3468. <https://doi.org/10.4049/jimmunol.174.6.3461>.
46. Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. 2010. Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infect Immun* 78:1601–1609. <https://doi.org/10.1128/IAI.01171-09>.
47. Wolf JM, Rivera J, Casadevall A. 2012. Serum albumin disrupts *Cryptococcus neoformans* and *Bacillus anthracis* extracellular vesicles. *Cell Microbiol* 14:762–773. <https://doi.org/10.1111/j.1462-5822.2012.01757.x>.
48. Almeida F, Wolf JM, da Silva TA, DeLeon-Rodriguez CM, Rezende CP, Personi AM, Fernandes FF, Silva-Rocha R, Martinez R, Rodrigues ML, Roque-Barreira MC, Casadevall A. 2017. Galectin-3 impacts *Cryptococcus neoformans* infection through direct antifungal effects. *Nat Commun* 8:1968. <https://doi.org/10.1038/s41467-017-02126-7>.
49. Bielska E, Sisquella MA, Aldeieg M, Birch C, O'Donoghue EJ, May RC. 2018. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen *Cryptococcus gattii*. *Nat Commun* 9:1556. <https://doi.org/10.1038/s41467-018-03991-6>.
50. Nobile CJ, Johnson AD. 2015. *Candida albicans* biofilms and human disease. *Annu Rev Microbiol* 69:71–92. <https://doi.org/10.1146/annurev-micro-091014-104330>.
51. Kim J, Sudbery P. 2011. *Candida albicans*, a major human fungal pathogen. *J Microbiol* 49:171–177. <https://doi.org/10.1007/s12275-011-0164-7>.
52. Vargas G, Rocha JD, Oliveira DL, Albuquerque PC, Frases S, Santos SS, Nosanchuk JD, Gomes AM, Medeiros LC, Miranda K, Sobreira TJ, Nakayasu ES, Arigi EA, Casadevall A, Guimaraes AJ, Rodrigues ML, Freire-de-Lima CG, Almeida IC, Nimrichter L. 2015. Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. *Cell Microbiol* 17:389–407. <https://doi.org/10.1111/cmi.12374>.
53. Wolf JM, Espadas J, Luque-Garcia J, Reynolds T, Casadevall A. 2015. Lipid biosynthetic genes affect *Candida albicans* extracellular vesicle morphology, cargo, and immunostimulatory properties. *Eukaryot Cell* 14:745–754. <https://doi.org/10.1128/EC.00054-15>.
54. Kenno S, Speth C, Rambach G, Binder U, Chatterjee S, Caramalho R, Haas H, Lass-Flörl C, Shaughnessy J, Ram S, Gow NAR, Orth-Höller D, Würzner R. 2018. *Candida albicans* factor H binding molecule Hgt1p—a low glucose-induced transmembrane protein is trafficked to the cell wall and impairs phagocytosis and killing by human neutrophils. *Front Microbiol* 9:3319. <https://doi.org/10.3389/fmicb.2018.03319>.
55. Mendes RP, Cavalcante RS, Marques SA, Marques MEA, Venturini J, Sylvestre TF, Paniago AMM, Pereira AC, da Silva JF, Fabro AT, Bosco SMG, Bagagli E, Hahn RC, Levorato AD. 2017. Paracoccidioidomycosis: current perspectives from Brazil. *Open Microbiol J* 11:224–282. <https://doi.org/10.2174/1874285801711010224>.
56. Rodríguez Molina LM, Tobón Orozco AM. 2018. Paracoccidioidomycosis: global vision of a forgotten endemic mycosis. *Curr Trop Med Rep* 5:138–143. <https://doi.org/10.1007/s40475-018-0148-4>.
57. Leopold Wager CM, Wormley FL, Jr. 2014. Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. *Mucosal Immunol* 7:1023–1035. <https://doi.org/10.1038/mi.2014.65>.
58. da Silva TA, Roque-Barreira MC, Casadevall A, Almeida F. 2016. Extracellular vesicles from *Paracoccidioides brasiliensis* induced M1 polarization in vitro. *Sci Rep* 6:35867. <https://doi.org/10.1038/srep35867>.
59. Peres da Silva R, Heiss C, Black I, Azadi P, Gerlach JQ, Travassos LR, Joshi L, Kilcoyne M, Puccia R. 2015. Extracellular vesicles from *Paracoccidioides* pathogenic species transport polysaccharide and expose ligands for DC-SIGN receptors. *Sci Rep* 5:14213. <https://doi.org/10.1038/srep14213>.
60. Hatanaka O, Rezende CP, Moreno P, Freitas Fernandes F, Oliveira Brito PKM, Martinez R, Coelho C, Roque-Barreira MC, Casadevall A, Almeida F. 2019. Galectin-3 inhibits *Paracoccidioides brasiliensis* growth and impacts paracoccidioidomycosis through multiple mechanisms. *mSphere* 4:e00209-19. <https://doi.org/10.1128/mSphere.00209-19>.
61. Woods JP. 2016. Revisiting old friends: developments in understanding *Histoplasma capsulatum* pathogenesis. *J Microbiol* 54:265–276. <https://doi.org/10.1007/s12275-016-6044-5>.
62. Kroetz DN, Deepe GS. 2012. The role of cytokines and chemokines in *Histoplasma capsulatum* infection. *Cytokine* 58:112–117. <https://doi.org/10.1016/j.cyt.2011.07.430>.
63. Queiroz-Telles F, Fahal AH, Falci DR, Caceres DH, Chiller T, Pasqualotto AC. 2017. Neglected endemic mycoses. *Lancet Infect Dis* 17:e367–e377. [https://doi.org/10.1016/S1473-3099\(17\)30306-7](https://doi.org/10.1016/S1473-3099(17)30306-7).
64. Limper AH, Adenis A, Le T, Harrison TS. 2017. Fungal infections in HIV/AIDS. *Lancet Infect Dis* 17:e334–e343. [https://doi.org/10.1016/S1473-3099\(17\)30303-1](https://doi.org/10.1016/S1473-3099(17)30303-1).
65. Matos Baltazar L, Nakayasu ES, Sobreira TJ, Choi H, Casadevall A, Nimrichter L, Nosanchuk JD. 2016. Antibody binding alters the characteristics and contents of extracellular vesicles released by *Histoplasma capsulatum*. *mSphere* 1:e00085-15. <https://doi.org/10.1128/mSphere.00085-15>.
66. Baltazar LM, Zamith-Miranda D, Burnet MC, Choi H, Nimrichter L, Nakayasu ES, Nosanchuk JD. 2018. Concentration-dependent protein loading of extracellular vesicles released by *Histoplasma capsulatum* after antibody treatment and its modulatory action upon macrophages. *Sci Rep* 8:8065. <https://doi.org/10.1038/s41598-018-25665-5>.
67. Harada K, Saito M, Sugita T, Tsuboi R. 2015. *Malassezia* species and their associated skin diseases. *J Dermatol* 42:250–257. <https://doi.org/10.1111/1346-8138.12700>.
68. Nowicka D, Nawrot U. 2019. Contribution of *Malassezia* spp. to the development of atopic dermatitis. *Mycoses* 62:588–596. <https://doi.org/10.1111/myc.12913>.
69. Johansson HJ, Vallhov H, Holm T, Gehrman U, Andersson A, Johansson C, Blom H, Carroni M, Lehtio J, Scheynius A. 2018. Extracellular nanovesicles released from the commensal yeast *Malassezia sympodialis* are

- enriched in allergens and interact with cells in human skin. *Sci Rep* 8:9182. <https://doi.org/10.1038/s41598-018-27451-9>.
70. Zhang YJ, Han Y, Sun YZ, Jiang HH, Liu M, Qi RQ, Gao XH. 2019. Extracellular vesicles derived from *Malassezia furfur* stimulate IL-6 production in keratinocytes as demonstrated in in vitro and in vivo models. *J Dermatol Sci* 93:168–175. <https://doi.org/10.1016/j.jdermsci.2019.03.001>.
 71. Zhan P, Liu W. 2017. The changing face of dermatophytic infections worldwide. *Mycopathologia* 182:77–86. <https://doi.org/10.1007/s11046-016-0082-8>.
 72. Chakrabarti A, Bonifaz A, Gutierrez-Galhardo MC, Mochizuki T, Li S. 2015. Global epidemiology of sporotrichosis. *Med Mycol* 53:3–14. <https://doi.org/10.1093/mmy/myu062>.
 73. de Beer ZW, Duong TA, Wingfield MJ. 2016. The divorce of *Sporothrix* and *Ophiostoma*: solution to a problematic relationship. *Stud Mycol* 83:165–191. <https://doi.org/10.1016/j.simyco.2016.07.001>.
 74. Gremiao ID, Miranda LH, Reis EG, Rodrigues AM, Pereira SA. 2017. Zoonotic epidemic of sporotrichosis: cat to human transmission. *PLoS Pathog* 13:e1006077. <https://doi.org/10.1371/journal.ppat.1006077>.
 75. Nicola AM, Albuquerque P, Paes HC, Fernandes L, Costa FF, Kioshima ES, Abadio AKR, Bocca AL, Felipe MS. 2019. Antifungal drugs: new insights in research & development. *Pharmacol Ther* 195:21–38. <https://doi.org/10.1016/j.pharmthera.2018.10.008>.
 76. Nami S, Mohammadi R, Vakili M, Khezripour K, Mirzaei H, Morovati H. 2019. Fungal vaccines, mechanism of actions and immunology: a comprehensive review. *Biomed Pharmacother* 109:333–344. <https://doi.org/10.1016/j.biopha.2018.10.075>.
 77. De Serrano LO, Burkhart DJ. 2017. Liposomal vaccine formulations as prophylactic agents: design considerations for modern vaccines. *J Nanobiotechnology* 15:83. <https://doi.org/10.1186/s12951-017-0319-9>.
 78. Wang N, Chen M, Wang T. 2019. Liposomes used as a vaccine adjuvant-delivery system: from basics to clinical immunization. *J Control Release* 303:130–150. <https://doi.org/10.1016/j.jconrel.2019.04.025>.
 79. Vallejo MC, Nakayasu ES, Matsuo AL, Sobreira TJ, Longo LV, Ganiko L, Almeida IC, Puccia R. 2012. Vesicle and vesicle-free extracellular proteome of *Paracoccidioides brasiliensis*: comparative analysis with other pathogenic fungi. *J Proteome Res* 11:1676–1685. <https://doi.org/10.1021/pr200872s>.
 80. Tabora CP, Juliano MA, Puccia R, Franco M, Travassos LR. 1998. Mapping of the T-cell epitope in the major 43-kilodalton glycoprotein of *Paracoccidioides brasiliensis* which induces a Th-1 response protective against fungal infection in BALB/c mice. *Infect Immun* 66:786–793.
 81. Marques AF, da Silva MB, Juliano MA, Munhoz JE, Travassos LR, Tabora CP. 2008. Additive effect of P10 immunization and chemotherapy in anergic mice challenged intratracheally with virulent yeasts of *Paracoccidioides brasiliensis*. *Microbes Infect* 10:1251–1258. <https://doi.org/10.1016/j.micinf.2008.07.027>.
 82. Munoz JE, Luft VD, Amorim J, Magalhaes A, Thomaz L, Nosanchuk JD, Travassos LR, Tabora CP. 2014. Immunization with P10 peptide increases specific immunity and protects immunosuppressed BALB/c mice infected with virulent yeasts of *Paracoccidioides brasiliensis*. *Mycopathologia* 178:177–188. <https://doi.org/10.1007/s11046-014-9801-1>.
 83. Pitarch A, Jimenez A, Nombela C, Gil C. 2006. Decoding serological response to *Candida* cell wall immunome into novel diagnostic, prognostic, and therapeutic candidates for systemic candidiasis by proteomic and bioinformatic analyses. *Mol Cell Proteomics* 5:79–96. <https://doi.org/10.1074/mcp.M500243-MCP200>.
 84. Gil-Bona A, Llama-Palacios A, Parra CM, Vivanco F, Nombela C, Montelivola L, Gil C. 2015. Proteomics unravels extracellular vesicles as carriers of classical cytoplasmic proteins in *Candida albicans*. *J Proteome Res* 14:142–153. <https://doi.org/10.1021/pr500794a>.
 85. Pereira TC, de Barros PP, Fugisaki LRO, Rossoni RD, Ribeiro FC, de Menezes RT, Junqueira JC, Scorzoni L. 2018. Recent advances in the use of *Galleria mellonella* model to study immune responses against human pathogens. *J Fungi (Basel)* 4:E128. <https://doi.org/10.3390/jof4040128>.
 86. Specht CA, Lee CK, Huang H, Tipper DJ, Shen ZT, Lodge JK, Leszyk J, Ostroff GR, Levitz SM. 2015. Protection against experimental cryptococcosis following vaccination with glucan particles containing *Cryptococcus* alkaline extracts. *mBio* 6:e01905-15. <https://doi.org/10.1128/mBio.01905-15>.
 87. Specht CA, Lee CK, Huang H, Hester MM, Liu J, Luckie BA, Torres Santana MA, Mirza Z, Khoshkenar P, Abraham A, Shen ZT, Lodge JK, Akalin A, Homan J, Ostroff GR, Levitz SM. 2017. Vaccination with recombinant *Cryptococcus* proteins in glucan particles protects mice against cryptococcosis in a manner dependent upon mouse strain and cryptococcal species. *mBio* 8:e01872-17. <https://doi.org/10.1128/mBio.01872-17>.
 88. Chaturvedi AK, Hameed RS, Wozniak KL, Hole CR, Leopold Wager CM, Weintraub ST, Lopez-Ribot JL, Wormley FL, Jr. 2014. Vaccine-mediated immune responses to experimental pulmonary *Cryptococcus gattii* infection in mice. *PLoS One* 9:e104316. <https://doi.org/10.1371/journal.pone.0104316>.
 89. Colombo AC, Rella A, Normile T, Joffe LS, Tavares PM, Araujo GRDS, Frases S, Orner EP, Farnoud AM, Fries BC, Sheridan B, Nimrichter L, Rodrigues ML, Del Poeta M. 2019. *Cryptococcus neoformans* glucuronoxylomannan and sterylglucoside are required for host protection in an animal vaccination model. *mBio* 10:e02909-28. <https://doi.org/10.1128/mBio.02909-18>.
 90. Campos JH, Soares RP, Ribeiro K, Andrade AC, Batista WL, Torrecilhas AC. 2015. Extracellular vesicles: role in inflammatory responses and potential uses in vaccination in cancer and infectious diseases. *J Immunol Res* 2015:832057. <https://doi.org/10.1155/2015/832057>.
 91. Ochoa-Azze RF. 2018. Cross-protection induced by VA-MENGOC-BC(R) vaccine. *Hum Vaccin Immunother* 14:1064–1068. <https://doi.org/10.1080/21645515.2018.1438028>.
 92. Holst J, Oster P, Arnold R, Tatley MV, Næss LM, Aaberge IS, Galloway Y, McNicholas A, O'Hallahan J, Rosenqvist E, Black S. 2013. Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): lessons from past programs and implications for the future. *Hum Vaccin Immunother* 9:1241–1253. <https://doi.org/10.4161/hv.24129>.
 93. Petousis-Harris H. 2018. Impact of meningococcal group B OMV vaccines, beyond their brief. *Hum Vaccin Immunother* 14:1058–1063. <https://doi.org/10.1080/21645515.2017.1381810>.
 94. Coelho C, Casadevall A. 2019. Answers to naysayers regarding microbial extracellular vesicles. *Biochem Soc Trans* 47:1005–1012. <https://doi.org/10.1042/BST20180252>.

ANEXO C

Manuscrito: BRAUER, V. S.; PESSONI, A. M.; BITENCOURT, T. A.; DE PAULA, R. G.; DE OLIVEIRA ROCHA, L.; GOLDMAN, G. H.; ALMEIDA, F. Extracellular Vesicles from *Aspergillus flavus* Induce M1 Polarization In Vitro. mSphere, v.5, p.e00190-20, 2020.

Extracellular Vesicles from *Aspergillus flavus* Induce M1 Polarization *In Vitro*

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ABSTRACT *Aspergillus flavus*, a ubiquitous and saprophytic fungus, is the second most common cause of aspergillosis worldwide. Several mechanisms contribute to the establishment of the fungal infection. Extracellular vesicles (EVs) have been described as “virulence factor delivery bags” in several fungal species, demonstrating a crucial role during the infection. In this study, we evaluated production of *A. flavus* EVs and their immunomodulatory functions. We verified that *A. flavus* EVs induce macrophages to produce inflammatory mediators, such as nitric oxide, tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and IL-1 β . Furthermore, the *A. flavus* EVs enhance phagocytosis and killing by macrophages and induce M1 macrophage polarization *in vitro*. In addition, a prior inoculation of *A. flavus* EVs in *Galleria mellonella* larvae resulted in a protective effect against the fungal infection. Our findings suggest that *A. flavus* EVs are biologically active and affect the interaction between *A. flavus* and host immune cells, priming the innate immune system to eliminate the fungal infection. Collectively, our results suggest that *A. flavus* EVs play a crucial role in aspergillosis.

IMPORTANCE Immunocompromised patients are susceptible to several fungal infections. The genus *Aspergillus* can cause increased morbidity and mortality. Developing new therapies is essential to understand the fungal biology mechanisms. Fungal EVs carry important virulence factors, thus playing pivotal roles in fungal pathophysiology. No study to date has reported EV production by *Aspergillus flavus*, a fungus considered to be the second most common cause of aspergillosis and relevant food contaminator found worldwide. In this study, we produced *A. flavus* EVs and evaluated the *in vitro* immunomodulatory effects of EVs on bone marrow-derived macrophages (BMDMs) and *in vivo* effects in a *Galleria mellonella* model.

KEYWORDS *Aspergillus flavus*, extracellular vesicles, macrophage, cytokine, phagocytosis, killing, polarization, *Galleria mellonella*

Aspergillus flavus is a ubiquitous and saprophytic fungus (1). It is the second most common cause of invasive and noninvasive aspergillosis (infection caused by *Aspergillus* species) (2, 3). Furthermore, *A. flavus* can contaminate several crops, such as maize, peanuts, and cottonseed, both pre- and postharvest and can cause huge economic losses (2, 4). Ingestion of the fungus-contaminated food can be fatal, mainly due to the mycotoxin production; for example, aflatoxin B1 is a natural carcinogenic compound that affects the liver, resulting in hepatocellular carcinoma, and causes acute aflatoxicosis (5, 6). Immunocompetent individuals rarely suffer from aspergillosis. Nevertheless, in immunocompromised hosts (such as patients with neutropenia or receiving cytotoxic and corticosteroid therapy, undergoing transplantation, and suffer-

Citation Brauer VS, Pessoni AM, Bitencourt TA, de Paula RG, de Oliveira Rocha L, Goldman GH, Almeida F. 2020. Extracellular vesicles from *Aspergillus flavus* induce M1 polarization *in vitro*. mSphere 5:e00190-20. <https://doi.org/10.1128/mSphere.00190-20>.

Editor Aaron P. Mitchell, University of Georgia

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Received 27 February 2020

Accepted 19 April 2020

Published 6 May 2020

FIG 1 Extracellular vesicles (EVs) produced by *Aspergillus flavus*. Nanoparticle-tracking analysis of EVs isolated from *A. flavus* culture supernatant was performed using NanoSight NS300. (A) Representative histogram depicting the particle-size distribution and concentration of EV profiles from *A. flavus* (EVs $\times 10^8$ particles/ml). (B) Representative graphic of EV average sizes from 12 independent experiments. (C) Screenshot from the video recorded by NanoSight NS300, presenting EV distribution.

ing from HIV [human immunodeficiency virus] infection) (8), the aspirated conidia may germinate and colonize the tissue, resulting in the clinical effects of the disease (9). Recently, *A. flavus* was ranked among the 10 most highly pathogenic fungi worldwide (10).

Extracellular vesicles (EVs) are structures produced by all life domains; they range in size from 30 to 1,000 nm and are surrounded by a lipid bilayer, carry protein, lipids, polysaccharides, nucleic acids, and pigments, and are crucial in cell communication, physiology, and immunopathogenesis of fungal infections (11–13). Fungal EV cargo may influence the host-parasite relationship during fungal infections (14). In 2007, Rodrigues et al., studying *Cryptococcus neoformans*, initially reported production of EVs by fungi (15). Since then, fungal EV production has been described in several fungal species, such as *Cryptococcus gattii* (16), *Saccharomyces cerevisiae* (17), *Alternaria infectoria* (18), *Paracoccidioides brasiliensis* (19), *Histoplasma capsulatum* (20), *Candida albicans* (20), *Candida parapsilosis* (20), *Sporothrix schenckii* (20), *Sporothrix brasiliensis* (21), *Malassezia sympodialis* (22), *Pichia fermentans* (23), *Trichophyton interdigitale* (24), *Trichoderma reesei* (25), *Aspergillus fumigatus* (26), and others.

Considering the *Aspergillus* species, EV production was reported recently in *A. fumigatus* (26); however, no study has reported EV production by *A. flavus*. Thus, the present study aimed to evaluate whether *A. flavus* produces EVs and, if so, whether these EVs are able to stimulate an immune response of macrophages. Here, we demonstrated EV production from *A. flavus* and that these EVs induce bone marrow-derived macrophages to produce inflammatory mediators and fungicidal activities against conidia. Furthermore, EVs stimulated the M1 phenotype for macrophage polarization.

RESULTS

EVs from *A. flavus*. To verify whether *A. flavus* is able to produce EVs, the supernatant was obtained from conidium culture and EVs were purified. Through nanoparticle tracking analysis (NTA), we determined the size and distribution profile of *A. flavus* EVs (Fig. 1). These EVs ranged in size from 40 to 400 nm (Fig. 1A), with an average size of 116 nm (± 8.35 nm) and median of 114.6 nm from several cultures (Fig. 1). The mode of the diameter of most vesicles was approximately of 83.4 nm. The size and distribution profile of these EVs are illustrated in Fig. 1C via a screenshot of a video recorded by a NanoSight NS300 system.

***A. flavus* EVs induce proinflammatory mediators in BMDMs.** To evaluate the influence of *A. flavus* EVs on the host immune response profile, we analyzed whether these EVs stimulated the macrophages. Bone marrow-derived macrophages (BMDMs) were incubated with different concentrations of EVs (10^3 to 10^7 particles/ml), with medium alone, or with lipopolysaccharide (LPS) (1 μ g/ml) plus gamma interferon (IFN- γ) (2 ng/ml) for 48 h to investigate the production of cytokines and NO. Our findings suggest that *A. flavus* EVs induce the production of important inflammatory

FIG 2 EVs from *A. flavus* induce the production of inflammatory mediators by bone marrow-derived macrophages (BMDMs). BMDMs obtained from C57BL/6 mice were cultured with different concentrations of EVs (10⁴ to 10⁷ particles/ml) for 48 h at 37°C. As positive and negative controls, the BMDMs were treated with lipopolysaccharide (LPS, 1 μg/ml) plus gamma interferon (IFN-γ) (2 ng/ml) or medium only as indicated. The culture supernatant was used to quantify the levels of (A) tumor necrosis factor alpha (TNF-α), (B) nitrite, (C) interleukin-6 (IL-6), and (D) IL-1β using enzyme-linked immunosorbent assay (ELISA). Data represent results from three independent experiments. One-way ANOVA and Bonferroni's multiple-comparison tests were used for analysis of TNF-α and IL-6 data, and Kruskal-Wallis and Dunn's multiple-comparison tests were used for analysis of nitrite and IL-1β data. *, *P* < 0.05.

mediators by macrophages (Fig. 2). The production of tumor necrosis factor alpha (TNF-α) (Fig. 2A), NO (Fig. 2B), interleukin-6 (IL-6) (Fig. 2C), and IL-1β (Fig. 2D) occurred in a dose-dependent manner; however, the IL-10 levels in the BMDMs treated with *A. flavus* EVs were as low as in the nonstimulated cells (data not shown).

***A. flavus* EVs enhanced fungicidal activity of macrophages.** Since phagocytosis and killing are important effector functions in macrophages (27), we determined the phagocytic index of BMDMs and investigated whether EVs promote *A. flavus* fungicidal activity by macrophages. As shown in Fig. 3A, BMDMs stimulated with EVs presented enhanced conidium engulfment (55.6% more conidia) compared with the cells cultured

FIG 3 *A. flavus* EVs stimulate microbicidal activity of BMDMs. (A) BMDMs were plated on glass coverslips and cultured with EVs (10⁷ particles/ml) for 30 min and were treated with *A. flavus* conidia (macrophages/conidia = 1:1) for 4 h at 37°C, and the phagocytic index was determined. (B) BMDM previously treated with EVs (10⁷ particles/ml), for 30 min, were infected with *A. flavus* conidia (macrophages/conidia = 1:1) for 48 h at 37°C. The cells were lysed, and the lysate was plated to detect the viable fungi based on CFU counting technique. Data represents results from three independent experiments. For both phagocytosis and killing assays, the IFN-γ-containing medium and medium only were used as positive and negative controls, respectively. An unpaired, two-tailed *t* test was used from both analyses. *, *P* < 0.05.

FIG 4 EVs induce M1 polarization of BMDMs. BMDMs were cultured with 10^7 particles/ml for 6 h. The cells were further treated with IFN- γ (2 ng/ml) plus IL-12p40 (50 ng/ml) as a positive control for M1 phenotype or with IL-4 (50 ng/ml) plus IL-10 (50 ng/ml) as a positive control of M2 phenotype or with medium only as a negative control. The total RNA was extracted from the macrophages and converted into cDNA, and qRT-PCR analysis was performed to evaluate the relative expression levels of classical markers of macrophage polarization. (A) Inducible nitric oxide synthase (iNOS). (B) YM1. (C) Arginase-1. Data represents results from three independent experiments. An unpaired, two-tailed t test was used for iNOS, and an unpaired, two-tailed t test and Mann-Whitney test were used for YM1 and arginase. *, $P < 0.05$.

only with the medium. This percentage was also higher than the positive-control IFN- γ , with a 35.2% increase compared to the medium condition. Furthermore, we evaluated the fungicidal activity by using killing assays. We performed the phagocytosis assay, and additionally, we treated BMDMs with fungal conidia (ratio of macrophages/conidia = 1:1) for 48 h. The lysate was plated, and the viable fungal cells were enumerated. As depicted in Fig. 3B, the CFU rates from lysate BMDMs stimulated with either IFN- γ or EVs were lower than those seen under the medium condition. These results suggest that *A. flavus* EVs promote the uptake of *A. flavus* and enhance the fungicidal activity of BMDMs.

A. *flavus* EVs induce the macrophage M1 phenotype. BMDMs stimulated with *A. flavus* EVs produced proinflammatory mediators and enhanced phagocytosis and killing rates. These results suggest macrophage polarization to the “classical activated M1” phenotype. To confirm this hypothesis, BMDMs were treated with EVs (10^7 particles/ml) or IFN- γ plus IL-12p40 (positive control for M1 polarization), IL-4 plus IL-10 (positive control for M2 polarization), or medium only for 6 h, and quantification of the relative levels of the transcripts of polarization markers (inducible nitric oxide synthase [iNOS], YM1, and arginase-1) was performed. The iNOS (M1 marker) mRNA level was increased by 62-fold in the EV-stimulated BMDMs, representing a response higher than that seen with the positive control (IFN- γ plus IL-12p40) (Fig. 4A). Nevertheless, the mRNA levels of M2 polarization markers (arginase-1 and YM1) seen under conditions of EV stimulation remained close to the mRNA levels of the nonstimulated BMDMs. These data suggest that *A. flavus* EVs promote BMDM polarization toward the M1 phenotype.

A. *flavus* EVs reduce fungal burden in a *Galleria mellonella* model of infection by *A. flavus*. Since *A. flavus* EVs were able to stimulate killing activity from BMDMs, we decided to analyze the infection in a *G. mellonella* *in vivo* model. Prior to challenge, the larvae were treated with different concentrations of EVs (10^5 , 10^6 , and 10^7) or with phosphate-buffered saline (PBS) as a control. After 48 h, *A. flavus* conidia were inoculated in the larvae. As shown in Fig. 5A and B, stimulation of EVs resulted in dose-dependently decreased CFU levels and enhanced survival of the larvae, respectively. These results suggest that *A. flavus* EVs may prime the host innate immune system to eliminate the fungal infection.

DISCUSSION

This was the first study to demonstrate that *A. flavus* produce EVs. We have described the production and isolation of *A. flavus* EVs, the induction of proinflammatory mediators in stimulated macrophages with EVs, and enhanced fungicidal activity. Furthermore, *A. flavus* EVs increased the transcript levels of iNOS, a classical M1 polarization marker; however, they did not affect the YM1 and arginase-1 levels (M2

FIG 5 *A. flavus* EVs induce a protective effect on *Galleria mellonella* in an *in vivo* model. (A) *G. mellonella* larvae ($n = 5$ per group) were stimulated with *A. flavus* EVs (10^5 , 10^6 , and 10^7 particles) or with PBS as a control 48 h prior to infection with *A. flavus* conidia. Larvae were homogenized and levels of CFU content determined 48 h postinfection for fungal burden analysis. (B) Survival rates ($n = 5$ larvae per group) during 15 days postinfection. Data represent the results of two independent experiments. One-way ANOVA and Bonferroni's multiple-comparison tests were used for fungal burden analysis and the log rank (Mantel-Cox) test for survival curve analysis. *, $P < 0.05$.

polarization markers). We also demonstrated that EVs are biologically active using a *G. mellonella in vivo* model, in which prior stimulation of larvae with *A. flavus* EVs promoted decreased fungal burden and increased survival of larvae during the exposure to *A. flavus* conidia.

Considering the description of EV production from fungal species, these structures have been considered to be important carriers of biological compounds and are related to several functions such as pathogenicity, cell communication, and immunogenicity of fungal infections (12). Several studies previously described biologically active EVs produced by the fungal species (*T. interdigitale*, *P. brasiliensis*, *C. neoformans*, *C. albicans*, *M. sympodialis*, *S. brasiliensis*) (21, 22, 24, 28–32). Among the *Aspergillus* spp., EVs from *A. fumigatus* were able to stimulate TNF- α and chemokine (C-C motif) ligand 2 (CCL2) production by macrophages *in vitro* (26).

In accordance with our findings, EVs produced by *A. fumigatus* (26), *T. interdigitale* (24), *P. brasiliensis* (28), and *C. albicans* (29) were able to increase the effector activity of macrophages in different experimental models. In contrast, EVs from *H. capsulatum* decreased both the phagocytic activity and killing rate of BMDMs (33), suggesting an immunomodulatory activity of EVs. Depending on the pathogen, the fungal EVs can intensify or attenuate the progression of the infection (14).

Mechanisms involved in the relationship between EVs and immune system remain unclear. EVs carry tremendous molecular content, including molecules that interact with the pattern recognition receptors expressed on cells of the immune system (for example, receptors from family of C-type lectin), thereby resulting in activation of innate and/or adaptive responses and protection of the host against infection (34, 35). Thus, EVs may act as key modulators of the immune response for different fungal infections, thus demonstrating the potential of these structures as possible targets for immune interventions (14).

By inhalation, *A. flavus* conidia reach the respiratory tract and encounter the innate immune system cells, as alveolar macrophages (36), representing the first line of defense against the pulmonary fungal infection (37). Macrophages contain mechanisms to kill invader pathogens (such as phagocytosis, developed lysosomal compartment, cytokines, chemokines, and growth factor production) (38), as well as mechanisms of antigen processing and presentation in T cells, thus developing an adaptive immune response (39). Macrophages exhibit outstanding phenotypic plasticity to adapt their functions according to their microenvironment (40, 41), thereby altering their phenotype between the classically activated M1 and alternatively activated M2 phenotypes (42). While M2 macrophages are associated with such immunoregulatory functions and markers as inflammatory zone 1 (FIZZ-1), arginase-1, and chitinase-like YM1 (39, 40, 43),

M1 macrophages are inflammatory, tumoricidal, and microbicidal, having iNOS nitric oxide synthase 2 as a hallmark molecule (39, 40, 42, 44, 45). The switch between the M1 and M2 phenotypes may represent an important event that defines the progress of fungal infection (46).

During experimental infection by *A. flavus*, an inflammatory state is established on the mouse lungs, characterized by production of cytokines, including IFN- γ and IL-6, and by recruitment of the immune system cells (neutrophils, lymphocytes, and macrophages); however, at 12 h postinfection, decreased TNF- α levels and higher IL-10 concentrations are observed, suggesting an inflammatory form of regulation (47). Nevertheless, no studies have evaluated the macrophage polarization profile associated with infections by *A. flavus*. We have provided evidence about the inflammatory profile of macrophages stimulated with EVs, reflected by enhanced inflammatory mediator production and higher levels of phagocytosis and killing activities. Furthermore, the higher level of expression of iNOS and lower level of expression of YM1 and arginase-1 suggest M1 polarization. Similar M1 phenotype polarization results were also described previously in experiments in which BMDMs were stimulated with EVs from *T. interdigitale* (24). In *P. brasiliensis*, by reinforcing the M1 polarization, secondary stimulation of macrophages by EVs was able to induce the switch from the M2 phenotype to the M1 phenotype (28). Collectively, these data corroborate the ability of EVs to modulate the immune response and the relationship between the host and pathogen.

The M1 phenotype of macrophages plays a crucial role in fungal elimination. Both M1 development and M2 phenotype inhibition have been described as being protective against *A. fumigatus*, *C. neoformans*, and *H. capsulatum* infections (39, 48, 49). Thus, we hypothesize that M1 polarization stimulated by *A. flavus* EVs might favor fungal clearance. Taking advantage of the *G. mellonella* model, which functionally mimics the mammal innate immune system and displays important functions such as phagocytosis and reactive oxygen species production (50, 51), we demonstrated that *A. flavus* EVs were able to stimulate *G. mellonella* responses to *A. flavus* infection. This model had been used previously for pathogenicity analysis of different fungal species, such as *A. fumigatus* (52), *C. albicans* (53), and *C. neoformans* (54), including the effects of fungal EVs in *C. albicans* and *C. neoformans* (29, 55). Corroborating our results, prestimulation of *G. mellonella* with *C. albicans* EVs was also previously found to result in decreased CFU levels and increased survival of larvae (29). These data suggest that prestimulation of *G. mellonella* with EVs may prime the immune system and favors the fungal clearance. On the other hand, intramuscular *in vivo* injection in BALB/c mice of EVs from *Sporothrix brasiliensis* followed by subcutaneous fungal infection resulted in higher fungal burden and larger skin lesions than were seen with mice that did not receive EVs, suggesting that EVs may favor the establishment of *S. brasiliensis* infection (21). Despite the use of the different experimental models, the data collectively corroborate the view that EVs produced by fungal species are bioactive, playing immunomodulatory functions and influencing the pathogenesis of infection.

In this report, we have shown that *A. flavus* is able to produce and secrete EVs. These structures are immunogenic, stimulate microbicidal functions in macrophages, cause M1 polarization, and have protective effects against *A. flavus* infection. Our findings provide new evidence on *A. flavus* infection. EVs produced during the infection, with their immunomodulatory functions, could be suggested to be possible therapeutic targets to treat aspergillosis caused by *A. flavus*.

MATERIALS AND METHODS

Ethics statement. All experimental procedures using mice followed the Ethical Principles Guide for the Care and Use of Laboratory Animals adopted by the Brazilian College of Animal Experimentation. This study was approved by the Committee of Ethics in Animal Research of the Ribeirao Preto Medical School at the University of Sao Paulo, Brazil (RPMS-USP; protocol 242/2019).

***A. flavus* strain and culture conditions.** The *A. flavus* NRRL 6513 strain was used in all experiments. The strain was maintained in potato dextrose agar (PDA; BD, Sparks, MD, USA) for 5 to 7 days at 30°C for sporulation. For EV production, the conidia were enumerated using a Neubauer chamber, and we inoculated 2×10^7 spores on 400 ml of minimal medium {1% [wt/vol] glucose, 20 ml of a 20 $\times$ salt solution [120 g/liter NaNO₃, 10.4 g/liter KCl, 30 g/liter KH₂PO₄, 10.4 g/liter MgSO₄·7H₂O], 0.1% [vol/vol]

trace elements solution [22 g/liter ZnSO_4 , 11 g/liter H_3BO_3 , 5 g/liter MnCl_2 , 5 g/liter FeSO_4 , 1.6 g/liter CoCl_2 , 1.6 g/liter CuSO_4 , 1.1 g/liter $(\text{NH}_4)_2\text{MoO}_4$, 50 g/liter ethylenediaminetetraacetic acid (EDTA)] and the pH was adjusted to 6.5 with NaOH. The culture was maintained for 72 h at 200 rpm and 37°C.

Isolation, characterization, and quantification of EVs. The culture was isolated as described by Vallejo et al. (19) with slight modifications. After 72 h of culture in the minimal medium, the supernatant was obtained using sterile Miracloth (Millipore, Billerica, MA, USA) and was concentrated via the use of an Amicon ultrafiltration system (Millipore, Billerica, MA, USA) (100-kDa cutoff), centrifuged at $15,000 \times g$ for 15 min at 4°C, and ultracentrifuged at 60,000 rpm at 4°C for 1 h. The EVs were suspended in 500 μl of sterile nuclease-free water (Sigma-Aldrich, St. Louis, MO, USA).

The size and distribution of EVs from independent growth cultures were verified via nanoparticle tracking analysis (NTA) using a NanoSight NS300 system (Malvern Instruments, Malvern, United Kingdom) as previously described (24). The particle size, distribution, and quantification were carried out using NanoSight software (version 3.2.16).

Preparation of BMDMs. BMDMs were isolated as described by Marim et al. and Bitencourt et al. (24, 56), with slight modifications. Briefly, the bone marrow cells were obtained from the femur and tibia of adult (6 weeks of age), WT (wild-type), male C57BL/6 mice. To release the cells, the bone marrow was flushed with RPMI 1640 medium, and the obtained cells were cultured for 6 days using RPMI 1640 supplemented with 20% fetal cow serum and 30% L-929 cell-conditioned medium (as a source of macrophage/granulocyte colony-stimulating factor). The adherent cells were collected and enumerated using a Neubauer chamber. The BMDMs were plated in the presence of the following different stimuli: EVs (10^3 to 10^7 particles/ml), lipopolysaccharide (LPS; 1 $\mu\text{g/ml}$) plus gamma interferon ($\text{IFN-}\gamma$) (2 ng/ml) or $\text{IFN-}\gamma$ (2 ng/ml) plus interleukin-12p40 (IL-12p40) (50 ng/ml), IL-4 (50 ng/ml) plus IL-10 (50 ng/ml), or medium only. The cultures were maintained for 6 h for quantitative reverse transcription-PCR (qRT-PCR) analysis or for 48 h for quantification of cytokines and nitric oxide.

Measurement of nitric oxide (NO) production. BMDMs were plated on 48-well plates (1.5×10^6 cells/ml; 7.5×10^5 cells/well), and after 48 h, the supernatant was collected and used for NO production. NO production was measured as described by Green et al. (57). Briefly, 50 μl of the cell supernatant was incubated with the same volume of Griess reagent (1.0% sulfanilamide, 0.1% naphthalene diamine dihydrochloride, 2.5% H_3PO_4) for 10 min at room temperature. The content was analyzed at a wavelength of 550 nm on a microplate-scanning spectrophotometer (PowerWave-X; BioTek Instruments, Inc., Winooski, VT, USA). With a standard curve generated by using the known concentrations of NaNO_2 diluted on RPMI medium, the absorbance data were converted into NO concentration values (expressed as micromoles).

Cytokine measurement. The cytokine measurement was verified for the BMDM culture supernatant (48 h; 1.5×10^6 cells/ml; 7.5×10^5 cells/well in a 48-well plate). The cytokines (tumor necrosis factor alpha [TNF- α], IL-6, IL-1 β , and IL-10) were quantified using an enzyme-linked immunosorbent assay (ELISA) kit, according to the protocol of the manufacturer (BD Biosciences, Pharmingen, San Diego, CA, USA). The concentrations were calculated using standard curves prepared with murine recombinant cytokines, and the sample absorbance was read at 450 nm using a microplate-scanning spectrophotometer (PowerWave-X; BioTek Instruments, Inc., Winooski, VT, USA).

qRT-PCR analysis. After 6 h of BMDM culture (2×10^6 cells/ml; 1×10^6 cells/well; 24-well plates), the cells were used to obtain the total RNA content using TRIzol reagent (Invitrogen, Life Technologies, Camarillo, CA, USA), and the protocol was performed in accordance with the manufacturer's instruction. The RNA was subjected to reverse transcription on cDNA, applying an ImProm-II reverse transcription system (Promega, Fitchburg, WI, USA) using oligo(dT). The qRT-PCR and the primers used were as described by da Silva et al. (28). The transcript levels were analyzed, and β -actin was used as an endogenous control.

Phagocytosis and killing assay. To obtain the phagocytic index, 2×10^5 cells/well were plated on glass 13-mm-diameter coverslips placed on 24-well plates (Dulbecco's modified Eagle medium [DMEM] with 10% fetal bovine serum [FBS]). The cells were treated with $\text{IFN-}\gamma$ (50 ng/ml), EVs (10^7 vesicles/ml), or medium alone for 30 min. Thereafter, the cells were washed with phosphate-buffered saline (PBS), and BMDMs were treated with conidia of *A. flavus* (2×10^5 conidia; macrophages/conidia = 1:1) for 4 h at 37°C with 5% CO_2 . Furthermore, the glass coverslips were washed with PBS and stained with Giemsa. An average of 100 macrophages was enumerated to determine the percentage of conidia that were ingested per macrophage.

For the killing assay, 5×10^5 cells/well were plated on a 24-well plate and treated under conditions similar to those described for the phagocytosis assay. Next, the BMDMs were treated with 5×10^5 conidia of *A. flavus* (macrophages/conidia = 1:1) for 48 h, in DMEM plus 10% FBS, at 37°C and 5% CO_2 . The culture supernatant was discarded after centrifugation (3,500 rpm, 10 min), and the cells were washed with PBS, followed by lysis with cold water. A serial dilution was prepared using the lysate, and the cells were then plated on PDA. The plates were incubated at 37°C for 48 h. The viable fungi were enumerated, and the CFU counts per milliliter were calculated.

Fungal burden assay and *Galleria mellonella* survival. Fungal burden and *G. mellonella* survival assays were performed as described previously (6, 29, 58) with slight modifications. Briefly, 10 larvae per group were selected that were similar in weight (approximately 275 to 330 mg) and without any gray coloring marks. Then, a 50- μl volume containing 10^5 , 10^6 , or 10^7 EVs was injected into the last left proleg, directly on hemocoel. PBS was used as a control. The larvae were maintained at 37°C in the dark for 48 h. Then, an *A. flavus* spore solution (1×10^4 spores/ml) was prepared, and a 10- μl volume was inoculated in all selected larvae, totaling 100 spores/larvae, at the same site of injection. The mortality rate of larvae ($n = 5$) was monitored daily during 15 days; the larvae that did not present movement after touch

stimulation were considered dead. Two days postinfection, larvae ($n = 5$) were homogenized on PBS, and the resulting solution was plated on PDA. The plates were incubated at 37°C, for 48 h, and CFU counts were determined.

Statistical analysis. The statistical analysis was performed using GraphPad Prism software version 8.0.1 (GraphPad Software, San Diego, CA). The experiments were carried out in triplicate (3 independent experiments), and the graphs present means $\pm$ SD (standard deviations). One-way analysis of variance (ANOVA) and Bonferroni's multiple-comparison tests were used when the data followed a normal distribution, and Kruskal-Wallis and Dunn's multiple-comparison tests were used for the nonparametric data. In addition, a two-tailed unpaired t test was also used for the parametric data and a two-tailed unpaired t test along with a Mann-Whitney test for nonparametric data. The *in vivo* experiments were performed in duplicate (two independent experiments), with 5 larvae per group for both experiments (*Galleria mellonella* survival assay and fungal burden assay). For comparisons of survival curves, log rank (Mantel-Cox) tests were used. For fungal burden, the graphed data are expressed as means $\pm$ SD, and the data were compared using one-way ANOVA and Bonferroni's multiple-comparison tests. P values of <0.05 were considered to be statistically significant, and all conditions were compared with the "medium" condition.

ACKNOWLEDGMENTS

We acknowledge Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, grant number 2016/03322-7), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

REFERENCES

1. Sugui JA, Kwon-Chung KJ, Juvvadi PR, Latgé J-P, Steinbach WJ. 2014. *Aspergillus fumigatus* and related species. Cold Spring Harb Perspect Med 5:a019786. <https://doi.org/10.1101/cshperspect.a019786>.
2. Pasqualotto AC. 2009. Differences in pathogenicity and clinical syndromes due to *Aspergillus fumigatus* and *Aspergillus flavus*. Med Mycol 47(Suppl 1):S261–S270. <https://doi.org/10.1080/13693780802247702>.
3. Almeida F, Rodrigues ML, Coelho C. 2019. The still underestimated problem of fungal diseases worldwide. Front Microbiol 10:214. <https://doi.org/10.3389/fmicb.2019.00214>.
4. Jackson SA, Dobson A. 2016. Yeasts and molds: *Aspergillus flavus*. Reference module in food science. Elsevier, Amsterdam, Netherlands. <https://doi.org/10.1016/B978-0-08-100596-5.01086-6>.
5. Amaike S, Keller NP. 2011. *Aspergillus flavus*. Annu Rev Phytopathol 49:107–133. <https://doi.org/10.1146/annurev-phyto-072910-095221>.
6. Alhmar JM, Puel O, Cary JW, Calvo AM. 6 March 2019, posting date. The *Aspergillus flavus* *rtfA* gene regulates plant and animal pathogenesis and secondary metabolism. Appl Environ Microbiol <https://doi.org/10.1128/AEM.02446-18>.
7. Reference deleted.
8. Stevens DA, Melikian GL. 2011. Aspergillosis in the 'nonimmunocompromised' host. Immunol Invest 40:751–766. <https://doi.org/10.3109/08820139.2011.614307>.
9. Hogan LH, Klein BS, Levitz SM. 1996. Virulence factors of medically important fungi. Clin Microbiol Rev 9:469–488. <https://doi.org/10.1128/CMR.9.4.469>.
10. Hyde KD, Al-Hatmi AMS, Andersen B, Boekhout T, Buzina W, Dawson TL, Eastwood DC, Jones EBG, de Hoog S, Kang Y, Longcore JE, McKenzie EHC, Meis JF, Pinson-Gadais L, Rathnayaka AR, Richard-Forget F, Stadler M, Theelen B, Thongbai B, Tsui A. 2018. The world's ten most feared fungi. Fungal Divers 93:161–194. <https://doi.org/10.1007/s13225-018-0413-9>.
11. Joffe LS, Nimrichter L, Rodrigues ML, Del Poeta M. 2016. Potential Roles of fungal extracellular vesicles during infection. mSphere 1:e00099-16. <https://doi.org/10.1128/mSphere.00099-16>.
12. Rodrigues ML, Casadevall A. 2018. A two-way road: novel roles for fungal extracellular vesicles. Mol Microbiol 110:11–15. <https://doi.org/10.1111/mmi.14095>.
13. Zamith-Miranda D, Nimrichter L, Rodrigues ML, Nosanchuk JD. 2018. Fungal extracellular vesicles: modulating host-pathogen interactions by both the fungus and the host. Microbes Infect 20:501–504. <https://doi.org/10.1016/j.micinf.2018.01.011>.
14. Freitas MS, Bonato VLD, Pessoa AM, Rodrigues ML, Casadevall A, Almeida F. 2019. Fungal extracellular vesicles as potential targets for immune interventions. mSphere 4:e00747-19. <https://doi.org/10.1128/mSphere.00747-19>.
15. Rodrigues ML, Nimrichter L, Oliveira DL, Frases S, Miranda K, Zaragoza O, Alvarez M, Nakouzi A, Feldmesser M, Casadevall A. 2007. Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot Cell 6:48–59. <https://doi.org/10.1128/EC.00318-06>.
16. Bielska E, Sisquella MA, Aldeieg M, Birch C, O'Donoghue EJ, May RC. 2018. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen *Cryptococcus gattii*. Nat Commun 9:1556. <https://doi.org/10.1038/s41467-018-03991-6>.
17. Oliveira DL, Nakayasu ES, Joffe LS, Guimarães AJ, Sobreira TJP, Nosanchuk JD, Cordero RJB, Frases S, Casadevall A, Almeida IC, Nimrichter L, Rodrigues ML. 2010. Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. PLoS One 5:e11113. <https://doi.org/10.1371/journal.pone.0011113>.
18. Silva BMA, Prados-Rosales R, Espadas-Moreno J, Wolf JM, Luque-García JL, Gonçalves T, Casadevall A. 2014. Characterization of *Alternaria infectoria* extracellular vesicles. Med Mycol 52:202–210. <https://doi.org/10.1093/mmy/myt003>.
19. Vallejo MC, Matsuo AL, Ganiko L, Medeiros LC, Miranda K, Silva LS, Freymuller-Haapalainen E, Sinigaglia-Coimbra R, Almeida IC, Puccia R. 2011. The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic alpha-Galactosyl epitopes. Eukaryot Cell 10:343–351. <https://doi.org/10.1128/EC.00227-10>.
20. Albuquerque PC, Nakayasu ES, Rodrigues ML, Frases S, Casadevall A, Zancoppe-Oliveira RM, Almeida IC, Nosanchuk JD. 2008. Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. Cell Microbiol 10:1695–1710. <https://doi.org/10.1111/j.1462-5822.2008.01160.x>.
21. Ikeda MAK, de Almeida JRF, Jannuzzi GP, Cronemberger-Andrade A, Torrecilhas ACT, Moretti NS, da Cunha JPC, de Almeida SR, Ferreira KS. 2018. Extracellular vesicles from *Sporothrix brasiliensis* are an important virulence factor that induce an increase in fungal burden in experimental sporotrichosis. Front Microbiol 9:2286–2286. <https://doi.org/10.3389/fmicb.2018.02286>.
22. Gehrmann U, Qazi KR, Johansson C, Hulténby K, Karlsson M, Lundberg L, Gabrielsson S, Scheynius A. 2011. Nanovesicles from *Malassezia sympodialis* and host exosomes induce cytokine responses—novel mechanisms for host-microbe interactions in atopic eczema. PLoS One 6:e21480. <https://doi.org/10.1371/journal.pone.0021480>.
23. Leone F, Bellani L, Muccifora S, Giorgetti L, Bongioanni P, Simili M, Maserti B, Del Carratore R. 2018. Analysis of extracellular vesicles produced in the biofilm by the dimorphic yeast *Pichia fermentans*. J Cell Physiol 233:2759–2767. <https://doi.org/10.1002/jcp.25885>.
24. Bitencourt TA, Rezende CP, Quaresimin NR, Moreno P, Hatanaka O, Rossi

- A, Martinez-Rossi NM, Almeida F. 2018. Extracellular vesicles from the dermatophyte *Trichophyton interdigitale* modulate macrophage and keratinocyte functions. *Front Immunol* 9:2343. <https://doi.org/10.3389/fimmu.2018.02343>.
25. de Paula RG, Antonieto ACC, Nogueira KMV, Ribeiro LFC, Rocha MC, Malavazi I, Almeida F, Silva RN. 2019. Extracellular vesicles carry cellulases in the industrial fungus *Trichoderma reesei*. *Biotechnol Biofuels* 12:146. <https://doi.org/10.1186/s13068-019-1487-7>.
 26. Souza JAM, Baltazar LDM, Carregal VM, Gouveia-Eufrazio L, de Oliveira AG, Dias WG, Campos Rocha M, Rocha de Miranda K, Malavazi I, Santos DDA, Frézard FJG, de Souza DDG, Teixeira MM, Soriani FM. 2019. Characterization of *Aspergillus fumigatus* extracellular vesicles and their effects on macrophages and neutrophils functions. *Front Microbiol* 10:2008. <https://doi.org/10.3389/fmicb.2019.02008>.
 27. Arango Duque G, Descoteaux A. 2014. Macrophage cytokines: involvement in immunity and infectious diseases. *Front Immunol* 5:491–491. <https://doi.org/10.3389/fimmu.2014.00491>.
 28. da Silva TA, Roque-Barreira MC, Casadevall A, Almeida F. 2016. Extracellular vesicles from *Paracoccidioides brasiliensis* induced M1 polarization in vitro. *Sci Rep* 6:35867. <https://doi.org/10.1038/srep35867>.
 29. Vargas G, Rocha JDB, Oliveira DL, Albuquerque PC, Frases S, Santos SS, Nosanchuk JD, Gomes AMO, Medeiros L, Miranda K, Sobreira TJP, Nakayasu ES, Arigi EA, Casadevall A, Guimaraes AJ, Rodrigues ML, Freire-de-Lima CG, Almeida IC, Nimrichter L. 2015. Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. *Cell Microbiol* 17:389–407. <https://doi.org/10.1111/cmi.12374>.
 30. Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. 2010. Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infect Immun* 78:1601–1609. <https://doi.org/10.1128/IAI.01171-09>.
 31. Almeida F, Wolf JM, da Silva TA, DeLeon-Rodriguez CM, Rezende CP, Pessoni AM, Fernandes FF, Silva-Rocha R, Martinez R, Rodrigues ML, Roque-Barreira MC, Casadevall A. 2017. Galectin-3 impacts *Cryptococcus neoformans* infection through direct antifungal effects. *Nat Commun* 8:1968. <https://doi.org/10.1038/s41467-017-02126-7>.
 32. Hatanaka O, Rezende CP, Moreno P, Freitas Fernandes F, Oliveira Brito PKM, Martinez R, Coelho C, Roque-Barreira MC, Casadevall A, Almeida F. 2019. Galectin-3 inhibits *Paracoccidioides brasiliensis* growth and impacts paracoccidioidomycosis through multiple mechanisms. *mSphere* 4:e00209-19. <https://doi.org/10.1128/mSphere.00209-19>.
 33. Baltazar LM, Zamith-Miranda D, Burnet MC, Choi H, Nimrichter L, Nakayasu ES, Nosanchuk JD. 2018. Concentration-dependent protein loading of extracellular vesicles released by *Histoplasma capsulatum* after antibody treatment and its modulatory action upon macrophages. *Sci Rep* 8:8065–8065. <https://doi.org/10.1038/s41598-018-25665-5>.
 34. da Silva RP, Heiss C, Black I, Azadi P, Gerlach JQ, Travassos LR, Joshi L, Kilcoyne M, Puccia R. 2015. Extracellular vesicles from *Paracoccidioides* pathogenic species transport polysaccharide and expose ligands for DC-SIGN receptors. *Sci Rep* 5:14213. <https://doi.org/10.1038/srep14213>.
 35. Patin EC, Thompson A, Orr SJ. 2019. Pattern recognition receptors in fungal immunity. *Semin Cell Dev Biol* 89:24–33. <https://doi.org/10.1016/j.semcdb.2018.03.003>.
 36. Xu S, Shinohara ML. 2017. Tissue-resident macrophages in fungal infections. *Front Immunol* 8:1798. <https://doi.org/10.3389/fimmu.2017.01798>.
 37. Schaffner A, Douglas H, Braude AI, Davis CE. 1983. Killing of *Aspergillus* spores depends on the anatomical source of the macrophage. *Infect Immun* 42:1109–1115. <https://doi.org/10.1128/IAI.42.3.1109-1115.1983>.
 38. Varol C, Mildner A, Jung S. 2015. Macrophages: development and tissue specialization. *Annu Rev Immunol* 33:643–675. <https://doi.org/10.1146/annurev-immunol-032414-112220>.
 39. Leopold Wager CM, Wormley FL, Jr. 2014. Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. *Mucosal Immunol* 7:1023–1035. <https://doi.org/10.1038/mi.2014.65>.
 40. Mantovani A, Sica A, Locati M. 2005. Macrophage polarization comes of age. *Immunity* 23:344–346. <https://doi.org/10.1016/j.immuni.2005.10.001>.
 41. Atri C, Guerfali FZ, Laouini D. 19 June 2018, posting date. Role of human macrophage polarization in inflammation during infectious diseases. *Int J Mol Sci* <https://doi.org/10.3390/ijms19061801>.
 42. Wang Y, Smith W, Hao D, He B, Kong L. 2019. M1 and M2 macrophage polarization and potentially therapeutic naturally occurring compounds. *Int Immunopharmacol* 70:459–466. <https://doi.org/10.1016/j.intimp.2019.02.050>.
 43. Lee KY. 31 January 2019, posting date. M1 and M2 polarization of macrophages: a mini-review. *Med Biol Sci Eng* 2:1–5. <https://doi.org/10.30579/mbse.2019.2.1.1>.
 44. Xue Q, Yan Y, Zhang R, Xiong H. 2018. Regulation of iNOS on immune cells and its role in diseases. *Int J Mol Sci* 19:3805. <https://doi.org/10.3390/ijms19123805>.
 45. Freitas MS, Oliveira AF, da Silva TA, Fernandes FF, Goncalves RA, Almeida F, Roque-Barreira MC. 2016. Paracoccin Induces M1 Polarization of Macrophages via Interaction with TLR4. *Front Microbiol* 7:1003. <https://doi.org/10.3389/fmicb.2016.01003>.
 46. Salazar F, Brown GD. 2018. Antifungal innate immunity: a perspective from the last 10 years. *J Innate Immun* 10:373–397. <https://doi.org/10.1159/000488539>.
 47. Anand R, Tiwary BN. 2010. Th1 and Th2 cytokines in a self-healing primary pulmonary *Aspergillus flavus* infection in BALB/c mice. *Cytokine* 52:258–264. <https://doi.org/10.1016/j.cyto.2010.07.428>.
 48. Moreira AP, Cavassani KA, Hullinger R, Rosada RS, Fong DJ, Murray L, Hesson DP, Hogaboam CM. 2010. Serum amyloid P attenuates M2 macrophage activation and protects against fungal spore-induced allergic airway disease. *J Allergy Clin Immunol* 126:712–721.e7. <https://doi.org/10.1016/j.jaci.2010.06.010>.
 49. Müller U, Stenzel W, Köhler G, Werner C, Polte T, Hansen G, Schütze N, Straubinger RK, Blessing M, McKenzie ANJ, Brombacher F, Alber G. 2007. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. *J Immunol* 179:5367–5377. <https://doi.org/10.4049/jimmunol.179.8.5367>.
 50. Pereira TC, de Barros PP, Fugisaki LRO, Rossoni RD, Ribeiro FC, de Menezes RT, Junqueira JC, Scorsoni L. 27 November 2018, posting date. Recent advances in the use of *Galleria mellonella* model to study immune responses against human pathogens. *J Fungi (Basel)* <https://doi.org/10.3390/jof4040128>.
 51. Trevijano-Contador N, Zaragoza O. 2018. Immune response of *Galleria mellonella* against human fungal pathogens. *J Fungi (Basel)* 5:3. <https://doi.org/10.3390/jof5010003>.
 52. Alcazar-Fuoli L, Buitrago M, Gomez-Lopez A, Mellado E. 2015. An alternative host model of a mixed fungal infection by azole susceptible and resistant *Aspergillus* spp strains. *Virulence* 6:376–384. <https://doi.org/10.1080/21505594.2015.1025192>.
 53. Santos RB, Scorsoni L, Namba AM, Rossoni RD, Jorge AOC, Junqueira JC. 2019. *Lactobacillus* species increase the survival of *Galleria mellonella* infected with *Candida albicans* and non-*albicans* *Candida* clinical isolates. *Med Mycol* 57:391–394. <https://doi.org/10.1093/mmy/myy032>.
 54. Mylonakis E, Moreno R, El Khoury JB, Idrum A, Heitman J, Calderwood SB, Ausubel FM, Diener A. 2005. *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. *Infect Immun* 73:3842–3850. <https://doi.org/10.1128/IAI.73.7.3842-3850.2005>.
 55. Colombo AC, Rella A, Normile T, Joffe LS, Tavares PM, de S. Araújo DR, Frases S, Orner EP, Farnoud AM, Fries BC, Sheridan B, Nimrichter L, Rodrigues ML, Del Poeta M. 2019. *Cryptococcus neoformans* glucuronoxylomannan and sterylglucoside are required for host protection in an animal vaccination model. *mBio* 10:e02909-18. <https://doi.org/10.1128/mBio.02909-18>.
 56. Marim FM, Silveira TN, Lima DS, Jr, Zamboni DS. 2010. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PLoS One* 5:e15263. <https://doi.org/10.1371/journal.pone.0015263>.
 57. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 126:131–138. [https://doi.org/10.1016/0003-2697\(82\)90118-X](https://doi.org/10.1016/0003-2697(82)90118-X).
 58. Fuchs BB, O'Brien E, Khoury JBE, Mylonakis E. 2010. Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. *Virulence* 1:475–482. <https://doi.org/10.4161/viru.1.6.12985>.

ANEXO D

Manuscrito: STEENWYK, J. L.; LIND, A. L.; RIES, L. N. A.; DOS REIS, T. F.; SILVA, L. P.; ALMEIDA, F.; BASTOS, R. W.; FRAGA DA SILVA, T. F. C.; BONATO, V. L. D.; PESSONI, A. M.; RODRIGUES, F.; RAJA, H. A.; KNOWLES, S. L.; OBERLIES, N. H.; LAGROU, K.; GOLDMAN, G. H.; ROKAS, A. Pathogenic Allodiploid Hybrids of *Aspergillus* Fungi. CURRENT BIOLOGY, v.30, p.1 - 13, 2020.

Pathogenic Allodiploid Hybrids of *Aspergillus* Fungi

Graphical Abstract

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In Brief

Steenwyk, Lind, et al. identify clinical isolates of *Aspergillus latus*, an allodiploid species of hybrid origin. These isolates exhibit phenotypic heterogeneity in infection-relevant traits and are distinct from closely related species. The results suggest that allodiploid hybridization contributes to the evolution of filamentous fungal pathogens.

Highlights

- *Aspergillus latus* clinical isolates were previously misidentified as *A. nidulans*
- *A. latus* is an allodiploid that arose via hybridization of two *Aspergillus* species
- *A. latus* hybrid isolates exhibit heterogeneity for several infection-relevant traits
- *A. latus* is phenotypically distinct from parental and closely related species

Article

Pathogenic Allodiploid Hybrids of *Aspergillus* Fungi

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<https://doi.org/10.1016/j.cub.2020.04.071>

SUMMARY

Interspecific hybridization substantially alters genotypes and phenotypes and can give rise to new lineages. Hybrid isolates that differ from their parental species in infection-relevant traits have been observed in several human-pathogenic yeasts and plant-pathogenic filamentous fungi but have yet to be found in human-pathogenic filamentous fungi. We discovered 6 clinical isolates from patients with aspergillosis originally identified as *Aspergillus nidulans* (section *Nidulantes*) that are actually allodiploid hybrids formed by the fusion of *Aspergillus spinulosporus* with an unknown close relative of *Aspergillus quadrilineatus*, both in section *Nidulantes*. Evolutionary genomic analyses revealed that these isolates belong to *Aspergillus latus*, an allodiploid hybrid species. Characterization of diverse infection-relevant traits further showed that *A. latus* hybrid isolates are genomically and phenotypically heterogeneous but also differ from *A. nidulans*, *A. spinulosporus*, and *A. quadrilineatus*. These results suggest that allodiploid hybridization contributes to the genomic and phenotypic diversity of filamentous fungal pathogens of humans.

INTRODUCTION

Interspecific hybridization can result in the formation of new species that substantially differ in their genomic and phenotypic characteristics from either parental species. One common mechanism by which interspecific hybrids can originate is allopolyploidy, which merges and multiplies the parental species' chromosomes [1, 2]. Allopolyploid hybrids may be more similar to one parent in some traits, reflect both parents in others, or may differ from both in the rest. Hybrids' distinct phenotypic profiles mean that they can potentially colonize new habitats [3, 4], whereas their polyploidy means that they can quickly become reproductively isolated from both parental species, forming a new species in the process [2]. Allopolyploids are relatively

common in plants but are also found in several other lineages, including in animals [5] and fungi [6, 7].

Among fungi, the most well-known example of allopolyploidy is the whole-genome duplication in an ancestor of the baker's yeast *Saccharomyces cerevisiae* [8–10]. Importantly, allopolyploidy is known from both fungal pathogens of plants [6, 11] and of animals [12, 13]. For example, the crucifer crop pathogen *Verticillium longisporum* and *Verticillium dahliae* are allopolyploid hybrids, as is the onion pathogen *Botrytis allii* [6, 14, 15]. Among fungal pathogens that infect humans, allopolyploidy has been reported in the ascomycete budding yeasts *Candida metapsilosis* [16] and *Candida orthopsilosis* [17] and in the basidiomycete yeast *Cryptococcus neoformans* x *Cryptococcus deneoformans* [18]. To our knowledge, allopolyploidy

has never been reported in human pathogenic filamentous fungi.

Aspergillus-related diseases, collectively known as aspergillosis, are caused by various species in the *Aspergillus* genus of filamentous fungi [19]. Although the saprophytic and ubiquitous airborne species *Aspergillus fumigatus* (section *Fumigati*) is responsible for most infections, several other species are also pathogenic [20–23]. One such species is *A. nidulans* (section *Nidulantes*); even though rarely pathogenic, *A. nidulans* is of interest because it is a major cause of invasive aspergillosis infections in chronic granulomatous disease (CGD) patients [24]. CGD is a genetic disorder that compromises the ability of phagocytes to produce reactive oxygen species, which act as broad-range antimicrobial chemicals [24, 25]. Strikingly, among CGD patients, *A. nidulans* is harder to treat than the more common pathogen *A. fumigatus* [24, 26].

To gain insights into *A. nidulans* pathogenicity, we sequenced 9 clinical isolates that were originally identified as *A. nidulans* from patients with various pulmonary diseases, including 2 isolates from CGD patients. Two of these isolates belong to *A. nidulans* and have been described in detail elsewhere [27]. However, our phenotypic and genomic analyses showed that 6 of the remaining 7 isolates are not *A. nidulans* but rather allodiploid hybrids of *Aspergillus latus*, a species that arose from allo-diploid hybridization between *Aspergillus spinulosporus* and an unknown second parental species closely related to *Aspergillus quadrilineatus* (both from section *Nidulantes*). Our analyses also revealed that the seventh isolate belongs to *A. spinulosporus*.

Phenotypic characterization of *A. latus* isolates, their parental species, and *A. nidulans* for diverse infection-relevant traits revealed two key findings. The first finding is that *A. latus* isolates exhibit strain heterogeneity for several infection-relevant traits. For example, we observed wide variation between *A. latus* isolates in their virulence in a disease model as well as in their interactions with human immune cells. The second finding is that *A. latus* isolates are phenotypically distinct from *A. spinulosporus*, *A. quadrilineatus*, and *A. nidulans*. For example, we found that *A. latus* hybrid spores are better at evading macrophage engulfment as well as evading hyphal killing and inhibition of germination by neutrophils than *A. nidulans* or *A. spinulosporus* and are more resistant to antifungals and oxidative stressors than *A. nidulans* and *A. quadrilineatus*. From a clinical perspective, our discovery of allodiploid fungal pathogens of humans suggests that accurate taxonomic identification of *Aspergillus* clinical isolates is a key first step to disease management. From an evolutionary perspective, our discovery suggests that allodiploid hybridization is a general mechanism of genomic and phenotypic diversification among human fungal pathogens.

RESULTS

Six Clinical Isolates Previously Characterized as *A. nidulans* Are Diploid

To gain insights into the genetic diversity of clinical isolates of *A. nidulans*, we analyzed 7 isolates from patients with different pulmonary diseases and compared them to haploid (A4) and the laboratory-induced diploid (R21/R153) reference strains of *A. nidulans* (Table 1). Using microscopy-based and/or molecular biology methods, all 7 isolates had previously been identified as

A. nidulans, all are similar in appearance when grown in standard laboratory conditions (Figure S1), and two were analyzed as *A. nidulans* isolates in previous experimental studies [28]. Examination of DNA content revealed that 6/7 isolates were more similar to the diploid *A. nidulans* R21/R153 strain than to the haploid *A. nidulans* A4 strain, suggesting that these 6 isolates are diploid (Figure 1A). The volume of asexual spores (conidia) is frequently proportional to the DNA content of the nucleus [29], and examination of their size showed that the same 6 isolates and the diploid *A. nidulans* strain have significantly larger spores than isolates with haploid genomes ($p < 0.001$, respectively; Dunn's test with Benjamini-Hochberg method of multiple test correction for both tests; Figure 1B).

To gain further insight into the genomes of the 6 diploid isolates and 1 haploid isolate, we sequenced them and compared their genome size and gene number with those of representative *Aspergillus* species known to be haploid (*A. clavatus* NRRL 1, *A. flavus* NRRL 3357, *A. fumigatus* Af293, *A. nidulans* A4, *A. niger* CBS 513.88, *A. sydowii* CBS 593.65, and *A. versicolor* CBS 583.65) [31–36]. We found that the genomes and gene numbers of the diploids were significantly larger (average genome size = 69.09 ± 5.68 Mb; average gene number = $21,321.57 \pm 2,342.13$) than those of the haploid representative *Aspergillus* species (average genome size = 32.62 ± 3.05 Mb; average gene number = $11,330.75 \pm 1,838.70$; Figure 1C; figshare image 1, 10.6084/m9.figshare.8114114; $p < 0.001$; Wilcoxon rank sum test for both tests; Table S1). Similarly, examination of gene content completeness revealed a significantly higher number of duplicated near-universally single-copy (BUSCO) genes in the diploids relative to representative *Aspergillus* species (Figure 1C; $p = 0.001$; Wilcoxon rank sum test). Thus, we concluded that 6/7 clinical isolates are diploids.

Diploid Clinical Isolates Are *Aspergillus latus*, a Species of Hybrid Origin

To examine the evolutionary origin of the clinical isolates, we retrieved their calmodulin and β -tubulin sequences and performed molecular phylogenetic analysis in the context of sequences of the two genes from all available taxa in the section *Nidulantes* phylogeny [37]. We found that the haploid clinical isolate 4060 had nearly identical calmodulin and β -tubulin sequences to other strains of *A. spinulosporus* and formed a monophyletic group with them, suggesting that it belongs to *A. spinulosporus* (Figure 2A; figshare: 10.6084/m9.figshare.8114114). Notably, we found that all 6 diploid clinical isolates contained two different copies of the calmodulin and β -tubulin genes; one copy was nearly identical to *A. spinulosporus* sequences, whereas the other was nearly identical to *A. latus* ones (Figure 2A; figshare: 10.6084/m9.figshare.8114114), raising the hypothesis that the diploids originated from interspecific hybridization between *A. spinulosporus* and *A. latus*.

To test this hypothesis, we analyzed the genome of *A. spinulosporus* strain NRRL 2395^T [30] and sequenced the type strain NRRL 200^T of *A. latus*. Examination of the DNA content and asexual spore size of these two species' genomes showed that *A. spinulosporus* NRRL 2395^T had similar values as clinical isolate 4060 (Figure 1) and was also placed in the same phylogenetic clade (Figure 2A); these findings confirm that clinical isolate 4060 belongs to *A. spinulosporus* and that

Table 1. Isolates Used in This Study

Strain/Isolate	Isolation Source	Reference/Source
<i>A. spinulosporus</i> NRRL 2395 ^T	soil, Buenos Aires, Argentina	[30]
<i>A. nidulans</i> A4	E. Yuill, Birmingham, UK	Fungal Genetics Stock Center
<i>A. quadrilineatus</i> NRRL 201 ^T	soil, New Jersey, USA	USDA
4060	clinical isolate from patient with chronic granulomatous disease	[28]
<i>A. latus</i> NRRL 200 ^T	unknown origin	USDA
MM151978	clinical isolate from patient with chronic obstructive pulmonary disease (COPD)	laboratory of Dr. Donald Sheppard, McGill University, Canada. Patient sex: unknown. Year isolated unknown.
NIH	clinical isolate from patient with chronic granulomatous disease	[28]
ASFU1710	clinical isolate from patient with primary immunodeficiency	sputum material, clinical laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. Patient sex: female. Isolated in year 2011
ASFU1908	clinical isolate from patient with asthma, allergic bronchopulmonary aspergillosis (ABPA), and IgG3 deficiency	sputum material, clinical laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. Patient sex: female. Isolated in year 2011. Isolated from the same patient as ASFU2033
ASFU2033	clinical isolate from patient with asthma, ABPA, and IgG3 deficiency	sputum material, clinical laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. Patient sex: female. Isolated from the same patient as ASFU1908 in year 2012

A. spinulosporus is one of the parental species involved in the interspecific hybridization event that gave rise to the 6 clinical isolates.

In contrast, the DNA content and spore size of the genome of the type strain of *A. latus* NRRL 200^T were similar to those of the 6 diploid clinical isolates (Figure 1). Furthermore, like the 6 clinical isolates, *A. latus* NRRL 200^T also had two copies of the calmodulin and β -tubulin gene sequences; one copy was nearly identical to *A. spinulosporus* sequences, and the other copy was closely related to but distinct from *A. quadrilineatus* sequences (Figure 2A; figshare: 10.6084/m9.figshare.8114114). These results suggest that the 6 diploid clinical isolates belong to *A. latus* and that *A. latus* is an allodiploid hybrid species that originated via interspecific hybridization between *A. spinulosporus* and a species closely related to *A. quadrilineatus*.

We tested this hypothesis by performing two different sets of analyses. In the first set of analyses, we sequenced, assembled, and annotated the genome of the type strain (NRRL 201^T) of *A. quadrilineatus*. Consistent with our hypothesis that *A. latus* is an allodiploid hybrid, we found that the *A. quadrilineatus* genome contains a single copy of the calmodulin and β -tubulin gene sequences, that these sequences form a monophyletic group with their orthologous sequences retrieved from the genome of a different *A. quadrilineatus* strain (strain CBS 853.96; <https://www.ncbi.nlm.nih.gov/sra/SRX5010607>), and that the *A. quadrilineatus* sequences form a sister group with one of the two sets of the *A. latus* sequences (Figure 2A).

In the second set of analyses, we estimated the sequence divergence of each gene in the genomes of the 7 clinical isolates as well as of *A. latus* NRRL 200^T and *A. quadrilineatus* NRRL 201^T from *A. spinulosporus* NRRL 2395^T. Under this analysis, the genomes of non-hybrids are expected to show a unimodal distribution (e.g., see control non-hybrid, *Zygosaccharomyces parvulus*; Figure 2Bi, left), whereas the genomes of hybrids are expected to show a bimodal distribution whose two modes correspond to the distributions of gene sequence divergence values from each parental genome (e.g., see control hybrid, *Zygosaccharomyces parvulus*; Figure 2Bi, right). We found that the haploid *A. spinulosporus* 4060 clinical isolate and *A. quadrilineatus* NRRL 201^T had unimodal distributions reflecting a history devoid of hybridization, and the 6 diploid clinical isolates and *A. latus* NRRL 200^T had bimodal distributions consistent with allodiploidy (Figure 2Bii). Furthermore, all 6 diploid isolates and *A. latus* NRRL 200^T contained nearly equal percentages of *A. spinulosporus* and

Table 1. Continued

Strain/Isolate	Isolation Source	Reference/Source
MO46149	clinical isolate from patient with myelodysplastic syndrome with pneumonia	sputum material, clinical laboratory of Dr. Catarina Lameiras, Department of Microbiology, Portuguese Oncology Institute of Porto, Porto, Portugal. Patient sex: male. Isolated in year 2011

The superscript “T” indicates that the strain is the type strain of the species. See also Figures 1 and S1 and Table S3.

Figure 1. Six Clinical Isolates Previously Characterized as *Aspergillus nidulans* and the Type Strain of *Aspergillus latus* Are Diploids

(A) Fluorescence-assisted cell sorting analysis suggests that the type strain of *Aspergillus latus* NRRL 200^T and 6 clinical isolates (MM151978, NIH, ASFU1710, ASFU1908, ASFU2033, and MO46149) previously identified as *Aspergillus nidulans* have diploid genomes. In contrast, *Aspergillus spinulosporus* NRRL2395 and clinical isolate 4060 have haploid genomes. The haploid *A. nidulans* strain A4 and the laboratory-induced diploid *A. nidulans* strain R21/R23 were used as references of haploid and diploid genomes, respectively.

(B) Asexual spore diameter is significantly different between the 6 diploid clinical isolates, the haploid *A. quadrilineatus*, *A. spinulosporus*, and *A. nidulans*, and the laboratory-induced diploid *A. nidulans* ($\chi^2 = 399.54$; $df = 2$; $p < 0.001$; Kruskal-Wallis rank sum test). Additional pairwise comparisons are shown by brackets; all comparisons used Dunn's test with Benjamini-Hochberg method of multi-test correction. *** $p \leq 0.001$. Boxplot hinges correspond to the first and third quartiles. Boxplot whiskers extend to values no greater than or less than 1.5 times the interquartile range. Data beyond this range are plotted individually.

(C) The 6 diploid clinical isolates and the *A. latus* NRRL 200^T strain have substantially larger genome sizes, gene numbers, and percent duplicated BUSCO genes compared to haploid genomes of representative *Aspergillus* species (*A. clavatus* NRRL 1, *A. flavus* NRRL 3357, *A. fumigatus* Af293, *A. nidulans* A4, *A. niger* CBS 513.88, *A. sydowii* CBS 593.65, and *A. versicolor* CBS 583.65). Genus and species names are abbreviated using the following scheme: *A. latus* (Alat); *A. spinulosporus* (Aspi); *A. quadrilineatus* (Aqua); and *A. nidulans* (Anid). CI represents clinical isolates. Dark gray represents *A. nidulans*, red represents *A. quadrilineatus*, blue represents *A. spinulosporus* and CI 4060, and purple represents *A. latus* and diploid isolates.

See also Tables 1 and S1–S3 and Figure S1.

A. quadrilineatus-like genes ($51.43\% \pm 0.74\%$ *A. spinulosporus*: $48.57\% \pm 0.74\%$ *A. quadrilineatus*-like; Figure 2B, pie charts), including nearly the full sets of *A. spinulosporus* and *A. quadrilineatus*-like secondary metabolic gene clusters (Table S2; figshare: 10.6084/m9.figshare.8114114). Putative homeologs exhibited an average nucleotide sequence divergence of $7.15\% \pm 0.03\%$, a value very similar to the average divergence of 7.14% observed between the 8,523 orthologs of *A. spinulosporus* NRRL 2395^T and *A. quadrilineatus* NRRL 201^T (Figure S2). These two sets of analyses confirm that the 6 diploid clinical isolates belong to *A. latus* and that *A. latus* is an allodiploid hybrid species that originated via interspecific hybridization between *A. spinulosporus* and a species closely related to *A. quadrilineatus*.

We next assessed whether the allodiploid hybrid species *A. latus* stems from a single hybridization event by comparing the genome-scale phylogenies constructed from the *A. spinulosporus* and the *A. quadrilineatus*-like parental genomes of the *A. latus* isolates (Figure S3). We found that the relationships of the *A. latus* isolates differed between the two phylogenies (Figure S3). This incongruence may stem from biological reasons (e.g., multiple hybridization events or recombination between the two parental genomes). However, the low level of support for relationships among isolates, especially in the phylogeny from the *A. spinulosporus* parental genome (Figure S3A),

means that we cannot exclude the possibility that the two phylogenies are not statistically significantly different. To test this, we evaluated whether the two topologies were statistically different using the approximately unbiased topology constraint test [38]. Using the *A. spinulosporus* data matrix, we found that we could not reject the topology inferred based on the *A. quadrilineatus*-like data matrix as statistically inferior; similarly, we could not reject the *A. spinulosporus* topology when we using the *A. quadrilineatus*-like data matrix ($p = 0.50$ for both tests). These results are consistent with the hypothesis that the two parental genomes of *A. latus* share the same evolutionary history.

To provide more insight on whether the two parental genomes *A. latus* hybrids undergo recombination, we first examined whether *A. latus* hybrids undergo the sexual cycle to produce sexual spores (ascospores). We found that all *A. latus* hybrids produce sexual spores and that the viability of these spores is similar to that of the sexual spores of their parental species (figshare image 2, 10.6084/m9.figshare.8114114). We next examined whether any contigs in the genomes of *A. latus* isolates had evidence of recombination events. Examination of long (≥ 100 kb) contigs revealed that most genes in most contigs contained genes from one or the other parent and that very few contigs contained substantial percentages of genes from both parents (Figure S4). For example, only an average of

Figure 2. The 6 Clinical Diploids Belong to *A. latus*, an Allodiploid Species Formed via Hybridization of *A. spinulosporus* and a Close Relative of *A. quadrilineatus*

(A) The type strain of *A. latus* NRRL 200^T and the 6 diploid clinical isolates have each two copies of the taxonomic markers β -tubulin and calmodulin. Phylogenetic analysis of their β -tubulin and calmodulin sequences together with sequences from representative taxa in section *Nidulantes* [37] suggests that clinical isolate 4060 belongs to *A. spinulosporus*, whereas *A. latus* NRRL 200^T and the 6 diploid clinical isolates are derived from two parental genomes. Interestingly, neither of the parental genomes is *A. nidulans*; rather, one is *A. spinulosporus* and the other is a species closely related to *Aspergillus quadrilineatus*. Newly sequenced isolates are shown in red and blue.

(B) Examination of sequence divergence (Ks; x axis) between each gene in an allodiploid and its best blast hit in *A. spinulosporus* confirms that the 6 diploid clinical isolates and the type strain of *A. latus* are allodiploid hybrids. In contrast, the 7th clinical isolate (4060) is a haploid *A. spinulosporus*. Similarly, we found no evidence of *A. quadrilineatus* NRRL 201^T forming via allodiploid hybridization. (Bi) Examination of the haploid, non-hybrid genome of *A. fumigatus* strain Af293 (negative control) shows a unimodal distribution, whereas examination of the diploid, hybrid genome *Zygosaccharomyces parabaillii* strain NBRC1047/ATCC56075 (Zpar) shows a bimodal distribution (positive control; gray represents genes from one parent; black represents genes from the other parent). Red represents genes assigned to the *A. quadrilineatus*-like parental genome; blue represents genes assigned to the *A. spinulosporus* parental genome. See also Figures S2–S4 and Data S1.

2.67% $\pm$ 0.71% contigs per *A. latus* hybrid genome contained substantial percentages of genes from both parental species (Figure S4). However, interpretation of these data is challenging for two reasons. First, the high sequence similarity of the two parental genomes means that identification of parent of origin

for highly conserved genes is difficult and likely explains the sporadic presence of one or a handful of genes from one parent in contigs comprised mostly of genes from the other parent. Second, alignment of several of the contigs that contain large numbers of genes from both parents to the *A. nidulans* A4

reference genome suggests that they are often patchworks of *A. nidulans* contigs; for example, a long stretch of an *A. latus* contig that matches one parent is homologous to *A. nidulans* chromosome 5 and the rest of the contig, which matches the other parent, is homologous to *A. nidulans* chromosome 7. The absence of *A. latus* contigs that contain genes from both parental species and map to a single *A. nidulans* chromosome suggests that *A. latus* contigs that contain genes from both parental species may stem from assembly artifacts. These results suggest that *A. latus* hybrids likely undergo little to no recombination between the two parental genomes.

The Genomes of the *A. latus* Allodiploid Hybrid Isolates Are Stable

To assess the genome stability of the *A. latus* isolates, we began by examining the gene content completeness of each parental genome. We found that each parental genome contained nearly all of the 1,315 BUSCO genes from the fungal phylum Ascomycota ($93.50\% \pm 1.88\%$ *A. spinulosporus* and $94.30\% \pm 0.40\%$ *A. quadrilineatus* like; figshare image 3, 10.6084/m9.figshare.8114114). Considering that gene content completeness from each parent is only slightly below that from haploid representative species (average = $96.33\% \pm 0.78\%$; min = 95.70% , *A. spinulosporus*; max = 97.3% , *A. nidulans* A4), these results suggest little loss of each parental genome by either aneuploidy or loss of heterozygosity events.

To further test this observation genome-wide, we examined the fraction of orthologous genes shared between the *A. spinulosporus* NRRL 2395^T strain and the parental genomes of *A. latus* isolates that stem from *A. spinulosporus*. We found that the *A. spinulosporus* parental genomes from *A. latus* hybrids shared a minimum of 9,227/9,611 orthologous genes with *A. spinulosporus* NRRL 2395^T; the sole exception was *A. latus* NRRL 200^T, which shared 8,749 orthologs (figshare: 10.6084/m9.figshare.8114114). Interestingly, the *A. spinulosporus* parental genome of *A. latus* NRRL 200^T shows by far the highest evolutionary rate in our phylogenomic analyses (Figure S3), suggesting that the *A. spinulosporus* parental genome of this strain may be more genetically unstable than those of the clinical isolates.

Examination of loss of heterozygosity and aneuploidy events in *A. latus* genomes revealed relatively little evidence for either. Two isolates contained loss of heterozygosity regions. The *A. latus* NRRL 200^T strain contained an ~1.2-Mb region homologous to the end of *A. nidulans* chromosome VIII that contained two copies of the *A. quadrilineatus*-like parental genome and lacked a copy of the *A. spinulosporus* genome. This region contains several BUSCO genes, which explains why this strain has a higher proportion of missing BUSCO genes from the *A. spinulosporus* parental genome compared to the 6 clinical isolates (figshare image 3, 10.6084/m9.figshare.8114114). The clinical isolate MO46149 contained an ~1-Mb region homologous to the beginning of *A. nidulans* chromosome V with two copies of the *A. spinulosporus* genome and lacked a copy of the *A. quadrilineatus*-like genome (Data S1). We did not find evidence for chromosome-scale aneuploidies (Data S1).

Lastly, by comparing the gene lengths of homeolog pairs as a signature of pseudogenization, we found evidence of pseudogenization in at least one gene among an average of $11.67\% \pm 0.004\%$ homeologs (Figure S2). These results suggest that the genomes of

the *A. latus* allodiploid hybrids are generally stable, that loss of heterozygosity is rare, that major aneuploidies have not occurred, and that both genes in ~88% of homeolog pairs are intact.

Hybrids Exhibit Wide Variation for Infection-Relevant Traits

To examine variation in infection-relevant traits between the hybrid isolates, one of their known parental species (*A. spinulosporus*), the closest known relative of their other parental species (*A. quadrilineatus*), and the species they were originally identified as (*A. nidulans*), we tested the virulence of all isolates in an invertebrate disease model and phenotypically characterized them across a wide variety of infection-relevant conditions, including interactions with host immune cells, drug susceptibility, oxidative stress, iron starvation, and temperature stress (Figures 3 and S5). Principal-component analysis (PCA) and examination of the traits with the greatest contributions to the observed variance among isolates revealed two major findings. First, the 7 *A. latus* hybrids exhibit substantial heterogeneity in their phenotypic profiles (Figure 3A). Second, the *A. latus* hybrids are phenotypically distinct from *A. nidulans* and their parental species but are more similar to *A. spinulosporus* than to *A. quadrilineatus* (Figure 3A). Among the traits tested, those with the largest contributions to the observed variation among isolates and species were interactions with host immune cells, antifungal drug susceptibility, and oxidative stress resistance (figshare image 3A and 4, 10.6084/m9.figshare.8114114). Here, we discuss exemplary phenotypic traits that highlight these two major findings (see Figure S5 for other phenotypes).

Phenotypic variation or strain heterogeneity among *A. latus* hybrids was observed for nearly every trait measured (Figures 3 and S5). For example, examination of virulence in the invertebrate greater wax moth (*Galleria mellonella*) model revealed substantial variation among isolates ($p < 0.001$; log rank test; Figure 3B). Specifically, we observed that *A. latus* isolate ASFU1710 was the most virulent and *A. latus* isolate MO46149 was the least virulent. Similarly, we found substantial strain heterogeneity in how much lytic and non-lytic NETosis (a process where neutrophils release neutrophil extracellular traps, or NETs, to kill microbes) [39] was stimulated by *A. latus* hybrid isolates (Figure 3C). For example, *A. latus* NIH did not substantially stimulate NETosis although *A. latus* ASFU1710 did. Strain heterogeneity was less pronounced for other traits, such as hyphal viability, drug susceptibility, and oxidative stress, yet all exhibited variation across isolates (Figures 3D–3F). One *A. latus* isolate that was consistently different from the rest is MO46149; for example, this isolate was twice as susceptible to hyphal killing by neutrophils compared to the other *A. latus* isolates and it was the isolate most sensitive to the antifungal caspofungin, as well as the isolate most tolerant to the oxidative stressor paraquat.

Phenotypic variation was also pronounced when we compared infection-relevant traits between *A. latus*, its two parental species, and *A. nidulans* (Figure 3A). For example, we found that *A. latus* hybrids (and *A. quadrilineatus*) were less susceptible to killing by neutrophils compared to *A. spinulosporus* (Figure 3D). In contrast, we found *A. latus* isolates (and *A. spinulosporus*) differed in their susceptibility to low doses of caspofungin (Figure 3E) or to high doses of oxidative stress (Figure 3F) from *A. quadrilineatus* and *A. nidulans*.

Figure 3. *A. latus* Hybrids Exhibit Strain Heterogeneity and Differ from Parental Species, *A. quadrilineatus*, and *A. nidulans* in Infection-Relevant Phenotypes

(A) Principal-component analysis of diverse infection-relevant phenotypes reveals strain heterogeneity among *A. latus* hybrids and that they differ from the closest relative of the unknown parent, *A. quadrilineatus*, and the *A. nidulans* A4 strain. Data for each phenotype were scaled prior to principal-component analysis. (B and C) Wide phenotypic variation among strains of *A. latus* hybrids as well as among the various species tested was observed for virulence in the *Galleria* moth model of disease (B) and for stimulation of NETosis, a process where neutrophils release neutrophil extracellular traps, or NETs, to kill microbes (C). (D) Examination of the percentage of hyphal viability between the various species revealed significant differences ($F(3) = 24.514$; $p < 0.001$; multi-factor ANOVA). (E) Examination of the caspofungin drug susceptibility profiles among *A. nidulans*, *A. spinulosporus*, and the *A. latus* revealed differences between the three species ($F(3) = 56.01$; $p < 0.001$; multi-factor ANOVA). At 1 µg/mL caspofungin treatment, *A. spinulosporus* and *A. latus* hybrids grew more than *A. nidulans* and *A. quadrilineatus* ($p < 0.001$ for both comparisons; Tukey honest significant differences test). We found no statistically significant differences between the various species at 8 µg/mL of caspofungin but observed a qualitative difference similar to growth in 1 µg/mL of caspofungin. (F) Examination of growth in the presence of the oxidative stress agent paraquat revealed differences among the various species ($F(3) = 30.25$; $p < 0.001$; multi-factor ANOVA).

Dark gray represents *A. nidulans*; red represents *A. quadrilineatus*; blue represents *A. spinulosporus*; and purple represents *A. latus*. All pairwise comparisons shown by brackets were examined using a Tukey honest significant differences test. $^{*}0.01 \leq p \leq 0.05$; $^{**}0.001 \leq p \leq 0.01$; $^{***}p \leq 0.001$. See also Figure S5. Barplots are displayed with error bars that correspond to one standard deviation from the mean.

In summary, we found substantial heterogeneity among *A. latus* hybrid isolates as well as between *A. latus* and closely related or parental species for diverse infection-relevant traits. Generally, *A. latus* hybrids are more similar to their known parent, *A. spinulosporus*, compared to the closest known relative of their other parent, *A. quadrilineatus*. Importantly, *A. latus* hybrids are also phenotypically distinct from *A. nidulans*, the species they were originally misdiagnosed as.

DISCUSSION

Infections by filamentous fungal pathogens affect hundreds of thousands of humans and exhibit very high mortality rates [40], so understanding the evolutionary mechanisms underlying their pathogenicity is of great interest. We have discovered several clinical isolates previously identified as *A. nidulans*, an important pathogen of CGD patients, which in reality are allopolyploid hybrids that arose via interspecific hybridization between *A. spinulosporus* and a close relative of *A. quadrilineatus* and belong to *A. latus*. In line with clinical misidentification of these species, *A. nidulans*, *A. spinulosporus*, *A. quadrilineatus*, and *A. latus* are known to be nearly indistinguishable with the exception of aspects of their ascospore micromorphology and their secondary metabolic profiles [37]. The allopolyploid hybrids show strain heterogeneity in their phenotypic profiles and differ from their parental species and *A. nidulans* with respect to several infection-relevant traits. Below, we discuss the implications of these results for disease management and the evolution of fungal pathogenicity.

Application of molecular typing, and more recently genomic, approaches to delineate fungal species and pathogens has revealed the existence of multiple, closely related species that are morphologically indistinguishable but genomically distinct from each other [41]. This “hidden” or “cryptic” genomic diversity is found in species from many genera that harbor major fungal pathogens, including *Aspergillus* [42–44]. Alarmingly, application of molecular methods on fungal clinical isolates has too begun to reveal that a significant portion of fungal infections are caused by these cryptic species. In the case of *Aspergillus*, studies in both the USA and Spain report that 10%–15% of aspergillosis infections stem from cryptic species [45, 46]. Understanding the biology of these cryptic species is essential for guidance in therapy, as many show high levels of antifungal drug resistance [45–47]. Several *A. fumigatus*-related cryptic species exhibit decreased susceptibility (relative to *A. fumigatus*) to antifungal drugs [48]; similarly, we found notable differences in certain phenotypic traits, including drug susceptibility, between the allopolyploid hybrids and *A. nidulans* (Figures 3E and S5).

A growing body of literature suggests that phenotypic heterogeneity among isolates of the same species is an under-appreciated factor in understanding fungal pathogenicity [49]. For example, several recent studies have identified phenotypic and genomic differences between *A. fumigatus* strains that are associated with virulence [50–52]; strain heterogeneity is also observed among *A. nidulans* strains [27]. In line with these studies, our work reveals considerable heterogeneity in infection-relevant traits among *A. latus* hybrids (Figures 3 and S5), further highlighting the importance of strain heterogeneity in understanding *Aspergillus* pathogenicity [49].

Allopolyploid hybrids typically have unstable genomes [12]. Evolutionary paths to achieve stability after hybridization include whole-genome duplication, total or partial chromosome loss, gene loss, and loss of heterozygosity [12]. For example, an ancient allopolyploid hybridization event in the budding yeast lineage that includes the baker's yeast *Saccharomyces cerevisiae* [9] was quickly followed by rapid gene loss in the parental genomes [53], with estimates suggesting that 10% of genes were lost in the first 10 Ma following hybridization [53]. Similar rates of gene loss have been reported in plants [54, 55] and animals [56], suggesting that rapid gene loss is a common outcome of hybridization. In contrast to these studies, we found that most *A. latus* hybrids (with the possible exception of *A. latus* NRRL 200^T) contain both copies of most homeolog gene pairs and have relatively stable genomes (Figures S2 and S4). Consistent with our genomic analyses, *A. latus* isolates exhibit minimal sectoring when grown in culture (Figure S1) and have similar ascospore viability compared to their closest relatives, *A. spinulosporus* and *A. quadrilineatus* (figshare image 2, 10.6084/m9.figshare.8114114). Furthermore, laboratory studies that have created synthetic *Aspergillus* hybrids—including those that created interspecies hybrids from distinct species in section *Nidulantes*—reported that some of these hybrids are relatively stable [57–60]. Taken together, these results suggest that *Aspergillus* hybrids may be more stable than other hybrid allopolyploids.

Although several examples of interspecies hybridization are known in fungi [6, 8–12, 14–18], most of them are ancient. Consequently, the steps that led to hybrid formation and maintenance are harder to elucidate. As the *A. latus* allopolyploid hybrids originated much more recently, and the mechanisms that underlie synthetic hybrid formation in *Aspergillus* have been extensively studied [57–60], we can propose a model to explain the origin and life cycle of *A. latus* (Figure 4). Under our model, the first step in the formation of the *A. latus* allopolyploid hybrid was the cellular fusion (or plasmogamy) of an *A. spinulosporus* parental isolate and an *A.-quadrilineatus*-like parental isolate through a parasexual or a sexual cycle. In the next step, the distinct nuclei contributed by the two parental isolates underwent nuclear fusion (or karyogamy) to create a single nucleus with a diploid genome comprised from the *A. spinulosporus* and *A.-quadrilineatus*-like genomes. Once formed, the allopolyploid hybrid species *A. latus* has been capable of undergoing both asexual and sexual reproduction and forms viable asexual spores (conidia; Figure 1) and sexual spores (ascospores; figshare image 2, 10.6084/m9.figshare.8114114S5).

Hybrids have been observed in several fungal pathogens of animals and plants [6, 12, 16, 18], suggesting that hybridization of pathogenic fungi poses threats to plant and animal health. Hybridization can result in the acquisition of new traits, such as host expansion or increased virulence. For example, two powdery mildew species that specialize in infecting different species of plants have been shown to hybridize and infect a plant species that neither parent can [61]. Similarly, hybridization is thought to contribute to virulence among human yeast pathogens, but clear examples are currently lacking [12]. To our knowledge, our results are the first report of hybrid clinical isolates in a filamentous fungal pathogen of humans (Figure 2). Like previous studies, we observe that the hybrids exhibit infection-relevant traits that differentiate them from their parental relatives and may provide a fitness

Figure 4. Proposed Model for the Evolution of *A. latus* via Allodiploid Hybridization

Under the model, haploid nuclei of an *A. spinulosporus* isolate and of an isolate from an *A. spinulosporus*-like species underwent cellular fusion (plasmogamy), forming a heterokaryotic mycelium (i.e., a mycelium where cells contain two distinct nuclei). Next, nuclear fusion (karyogamy) resulted in the merging of the two genetically distinct nuclei and their genomes into a single one, giving rise to the allodiploid species *A. latus*, which is capable of undergoing asexual and sexual reproduction to produce asexual spores (conidia) or sexual spores (ascospores).

advantage inside human hosts (Figure 3). However, it should be noted that the potential selection pressure for hybrids in patients is unknown; although we isolated the hybrids from patients with

diverse pulmonary diseases, we do not know whether the hybrids' primary lifestyle is that of a pathogen or whether they originated inside a host. Importantly, the type strain of *A. latus* (NRRL 200^T) is not a clinical isolate, suggesting that the species is also found in the environment. More broadly, the existence of hybrids in a diverse set of pathogenic fungi infecting a diverse set of animal and plant hosts raises the hypothesis that allodiploid hybridization contributes to the evolution and diversity of all kinds of fungal pathogens, perhaps to a greater extent than currently realized.

In summary, viewed from a medical mycology perspective, our discovery of allodiploid hybrid clinical isolates reveals the importance of accurate isolate identification and strain heterogeneity. Viewed from an evolutionary perspective, our and previous results suggest that hybridization contributes to the genomic and phenotypic diversification of filamentous fungal pathogens of humans and argue that hybridization represents a general mechanism that can be potentially employed by all fungal pathogens to adapt to all kinds of hosts.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cub.2020.04.071>.

ACKNOWLEDGMENTS

We thank members of the Rokas and Goldman laboratories, in particular Xing-Xing Shen, Matthew E. Mead, and Abigail L. Labella, for their helpful suggestions and discussions. We thank Drs. Catarina Lameiras and Donald C. Shepard for kindly sharing *Aspergillus* clinical isolates. This work was conducted in part using the resources of the Advanced Computing Center for Research and Education at Vanderbilt University (<http://www.accre.vanderbilt.edu/>). A.R. was supported in part by the National Science Foundation (DEB-1442113), the Vanderbilt Discovery Grant Program, the Burroughs Wellcome Fund, and the Guggenheim Foundation; J.L.S. and A.R. by the Howard Hughes Medical Institute through the James H. Gilliam Fellowships for Advanced Study program; A.L.L. by a US National Library of Medicine training grant (grant number 2T15LM007450); G.H.G. by the Brazilian São Paulo Research Foundation (FAPESP) (grant number 2016/07870-9) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); F.A. by FAPESP (grant number 2016/03322-7); L.N.A.R. by FAPESP (grant number 2017/14159-2); and F.R. by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER) (NORTE-01-0145-FEDER-000013).

AUTHOR CONTRIBUTIONS

J.L.S., A.L.L., G.H.G., and A.R. designed the research; J.L.S. and A.L.L. performed computational and statistical analyses; L.N.A.R., T.F.d.R., L.P.S., F.A., R.W.B., T.F.d.C.F.d.S., V.L.D.B., A.M.P., and F.R. performed experiments; K.L. contributed strains; H.A.R., S.L.K., and N.H.O. contributed strains and performed experiments; J.L.S. and A.L.L. prepared the figures with input from G.H.G. and A.R.; J.L.S., A.L.L., G.H.G., and A.R. wrote the manuscript; and all authors provided comments and input and have approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: July 16, 2019

Revised: February 25, 2020

Accepted: April 24, 2020

Published: June 4, 2020

REFERENCES

- Abbott, R., Albach, D., Ansell, S., Arntzen, J.W., Baird, S.J.E., Biernie, N., Boughman, J., Brelsford, A., Buerkle, C.A., Buggs, R., et al. (2013). Hybridization and speciation. *J. Evol. Biol.* 26, 229–246.
- Baack, E.J., and Rieseberg, L.H. (2007). A genomic view of introgression and hybrid speciation. *Curr. Opin. Genet. Dev.* 17, 513–518.
- Rieseberg, L.H., Raymond, O., Rosenthal, D.M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J.L., Schwarzbach, A.E., Donovan, A.K., and Lexer, C. (2003). Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* 301, 1211–1216.
- Rieseberg, L.H., Kim, S.-C., Randell, R.A., Whitney, K.D., Gross, B.L., Lexer, C., and Clay, K. (2007). Hybridization and the colonization of novel habitats by annual sunflowers. *Genetica* 129, 149–165.
- Mable, B.K., Alexandrou, M.A., and Taylor, M.I. (2011). Genome duplication in amphibians and fish: an extended synthesis. *J. Zool. (Lond.)* 284, 151–182.
- Depotter, J.R., Seidl, M.F., Wood, T.A., and Thomma, B.P. (2016). Interspecific hybridization impacts host range and pathogenicity of filamentous microbes. *Curr. Opin. Microbiol.* 32, 7–13.
- Dunn, B., and Sherlock, G. (2008). Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. *Genome Res.* 18, 1610–1623.
- Wolfe, K.H. (2015). Origin of the yeast whole-genome duplication. *PLoS Biol.* 13, e1002221.
- Marcel-Houben, M., and Gabaldón, T. (2015). Beyond the whole-genome duplication: phylogenetic evidence for an ancient interspecies hybridization in the baker's yeast lineage. *PLoS Biol.* 13, e1002220.
- Wolfe, K.H., and Shields, D.C. (1997). Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* 387, 708–713.
- Stukenbrock, E.H. (2016). The role of hybridization in the evolution and emergence of new fungal plant pathogens. *Phytopathology* 106, 104–112.
- Mixão, V., and Gabaldón, T. (2018). Hybridization and emergence of virulence in opportunistic human yeast pathogens. *Yeast* 35, 5–20.
- Lin, X., Patel, S., Litvintseva, A.P., Floyd, A., Mitchell, T.G., and Heitman, J. (2009). Diploids in the *Cryptococcus neoformans* serotype A population homozygous for the α mating type originate via unisexual mating. *PLoS Pathog.* 5, e1000283.
- Inderbitzin, P., Davis, R.M., Bostock, R.M., and Subbarao, K.V. (2011). The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS ONE* 6, e18260.
- Nielsen, K., and Yohalem, D.S. (2001). Origin of a polyploid botrytis pathogen through interspecific hybridization between *Botrytis aclada* and *B. byssoides*. *Mycologia* 93, 1064–1071.
- Pryszcz, L.P., Németh, T., Saus, E., Ksiezopolska, E., Hegedúsová, E., Nosek, J., Wolfe, K.H., Gacser, A., and Gabaldón, T. (2015). The genomic aftermath of hybridization in the opportunistic pathogen *Candida metapsilosis*. *PLoS Genet.* 11, e1005626.
- Schröder, M.S., Martínez de San Vicente, K., Prandini, T.H.R., Hammel, S., Higgins, D.G., Bagagli, E., Wolfe, K.H., and Butler, G. (2016). Multiple origins of the pathogenic yeast *Candida orthopsilosis* by separate hybridizations between two parental species. *PLoS Genet.* 12, e1006404.
- Rhodes, J., Desjardins, C.A., Sykes, S.M., Beale, M.A., Vanhove, M., Sakthikumar, S., Chen, Y., Gujja, S., Saif, S., Chowdhary, A., et al. (2017). Tracing genetic exchange and biogeography of *Cryptococcus neoformans* var. *grubii* at the global population level. *Genetics* 207, 327–346.
- Barnes, P.D., and Marr, K.A. (2006). Aspergillosis: spectrum of disease, diagnosis, and treatment. *Infect. Dis. Clin. North Am.* 20, 545–561, vi.
- van de Veerdonk, F.L., Gresnigt, M.S., Romani, L., Netea, M.G., and Latgé, J.-P. (2017). *Aspergillus fumigatus* morphology and dynamic host interactions. *Nat. Rev. Microbiol.* 15, 661–674.
- Brown, N.A., and Goldman, G.H. (2016). The contribution of *Aspergillus fumigatus* stress responses to virulence and antifungal resistance. *J. Microbiol.* 54, 243–253.
- Paulussen, C., Hallsworth, J.E., Álvarez-Pérez, S., Nierman, W.C., Hamill, P.G., Blain, D., Rediers, H., and Lievens, B. (2017). Ecology of aspergillosis: insights into the pathogenic potency of *Aspergillus fumigatus* and some other *Aspergillus* species. *Microb. Biotechnol.* 10, 296–322.
- Rokas, A., Mead, M.E., Steenwyk, J.L., Oberlies, N.H., and Goldman, G.H. (2020). Evolving moldy murderers: *Aspergillus* section *Fumigati* as a model for studying the repeated evolution of fungal pathogenicity. *PLoS Pathog.* 16, e1008315.
- Henriet, S.S.V., Verweij, P.E., and Warris, A. (2012). *Aspergillus nidulans* and chronic granulomatous disease: a unique host-pathogen interaction. *J. Infect. Dis.* 206, 1128–1137.
- Fang, F.C. (2011). Antimicrobial actions of reactive oxygen species. *MBio* 2, e00141-11.
- Dotis, J., and Roilides, E. (2004). Osteomyelitis due to *Aspergillus* spp. in patients with chronic granulomatous disease: comparison of *Aspergillus nidulans* and *Aspergillus fumigatus*. *Int. J. Infect. Dis.* 8, 103–110.
- Bastos, R.W., Valero, C., Silva, L.P., Schoen, T., Drott, M., Brauer, V., Silva-Rocha, R., Lind, A., Steenwyk, J.L., Rokas, A., et al. (2020). Functional characterization of clinical isolates of the opportunistic fungal pathogen *Aspergillus nidulans*. *MSphere* 5, e00153-20.
- Lee, M.J., Liu, H., Barker, B.M., Snarr, B.D., Gravelat, F.N., Al Abdallah, Q., Gavino, C., Baistrocchi, S.R., Ostapska, H., Xiao, T., et al. (2015). The fungal exopolysaccharide galactosaminogalactan mediates virulence by

- enhancing resistance to neutrophil extracellular traps. *PLoS Pathog.* **11**, e1005187.
29. Hfagy, F.C., and Roper, J.A. (1952). Deoxyribonucleic acid content of haploid and diploid *Aspergillus* conidia. *Nature* **170**, 713–714.
30. Steenwyk, J.L., Shen, X.-X., Lind, A.L., Goldman, G.H., and Rokas, A. (2019). A robust phylogenomic time tree for biotechnologically and medically important fungi in the genera *Aspergillus* and *Penicillium*. *MBio* **10**, e00925-19.
31. Fedorova, N.D., Khaldi, N., Joardar, V.S., Maiti, R., Amedeo, P., Anderson, M.J., Crabtree, J., Silva, J.C., Badger, J.H., Albarraq, A., et al. (2008). Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. *PLoS Genet.* **4**, e1000046.
32. Nierman, W.C., Yu, J., Fedorova-Abrams, N.D., Losada, L., Cleveland, T.E., Bhatnagar, D., Bennett, J.W., Dean, R., and Payne, G.A. (2015). Genome sequence of *Aspergillus flavus* NRRL 3357, a strain that causes aflatoxin contamination of food and feed. *Genome Announc.* **3**, e00168-15.
33. Nierman, W.C., Pain, A., Anderson, M.J., Wortman, J.R., Kim, H.S., Arroyo, J., Berriman, M., Abe, K., Archer, D.B., Bermejo, C., et al. (2005). Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438**, 1151–1156.
34. Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., Turner, G., de Vries, R.P., Albang, R., Albermann, K., et al. (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.* **25**, 221–231.
35. de Vries, R.P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Uchima, C.A., Anderluh, G., Asadollahi, M., Askin, M., Barry, K., et al. (2017). Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus *Aspergillus*. *Genome Biol.* **18**, 28.
36. Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.-J., Wortman, J.R., Batzoglou, S., Lee, S.-I., Baştürkmen, M., Spevak, C.C., Clutterbuck, J., et al. (2005). Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438**, 1105–1115.
37. Chen, A.J., Frisvad, J.C., Sun, B.D., Varga, J., Kocsubé, S., Dijksterhuis, J., Kim, D.H., Hong, S.-B., Houbraken, J., and Samson, R.A. (2016). *Aspergillus* section *Nidulantes* (formerly *Emericella*): polyphasic taxonomy, chemistry and biology. *Stud. Mycol.* **84**, 1–118.
38. Shimodaira, H. (2002). An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.* **51**, 492–508.
39. Branzk, N., Lubojemska, A., Hardison, S.E., Wang, Q., Gutierrez, M.G., Brown, G.D., and Papayannopoulos, V. (2014). Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* **15**, 1017–1025.
40. Brown, G.D., Denning, D.W., Gow, N.A., Levitz, S.M., Netea, M.G., and White, T.C. (2012). Hidden killers: human fungal infections. *Sci. Transl. Med.* **4**, 165rv13.
41. Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., and Fisher, M.C. (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genet. Biol.* **31**, 21–32.
42. Balajee, S.A., Gribskov, J.L., Hanley, E., Nickle, D., and Marr, K.A. (2005). *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryot. Cell* **4**, 625–632.
43. Geiser, D.M., Pitt, J.I., and Taylor, J.W. (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proc. Natl. Acad. Sci. USA* **95**, 388–393.
44. Pringle, A., Baker, D.M., Platt, J.L., Wares, J.P., Latgé, J.P., and Taylor, J.W. (2005). Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution* **59**, 1886–1899.
45. Perlin, D.S., Rautemaa-Richardson, R., and Alastruey-Izquierdo, A. (2017). The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect. Dis.* **17**, e383–e392.
46. Alastruey-Izquierdo, A., Alcazar-Fuoli, L., and Cuenca-Estrella, M. (2014). Antifungal susceptibility profile of cryptic species of *Aspergillus*. *Mycopathologia* **178**, 427–433.
47. Verweij, P.E., Ananda-Rajah, M., Andes, D., Arendrup, M.C., Brüggemann, R.J., Chowdhary, A., Cornely, O.A., Denning, D.W., Groll, A.H., Izumikawa, K., et al. (2015). International expert opinion on the management of infection caused by azole-resistant *Aspergillus fumigatus*. *Drug Resist. Updat.* **21–22**, 30–40.
48. Van Der Linden, J.W.M., Warris, A., and Verweij, P.E. (2011). *Aspergillus* species intrinsically resistant to antifungal agents. *Med. Mycol.* **49** (Suppl 1), S82–S89.
49. Keller, N.P. (2017). Heterogeneity confounds establishment of “a” model microbial strain. *MBio* **8**, e00135-17.
50. Kowalski, C.H., Beattie, S.R., Fuller, K.K., McGurk, E.A., Tang, Y.-W., Hohl, T.M., Obar, J.J., and Cramer, R.A., Jr. (2016). Heterogeneity among isolates reveals that fitness in low oxygen correlates with *Aspergillus fumigatus* virulence. *MBio* **7**, e01515-16.
51. Ries, L.N.A., Steenwyk, J.L., de Castro, P.A., de Lima, P.B.A., Almeida, F., de Assis, L.J., Manfio, A.O., Takahashi-Nakaguchi, A., Kusuya, Y., Hagiwara, D., et al. (2019). Nutritional heterogeneity among *Aspergillus fumigatus* strains has consequences for virulence in a strain- and host-dependent manner. *Front. Microbiol.* **10**, 854.
52. Kowalski, C.H., Kerkaert, J.D., Liu, K.-W., Bond, M.C., Hartmann, R., Nadell, C.D., Stajich, J.E., and Cramer, R.A. (2019). Fungal biofilm morphology impacts hypoxia fitness and disease progression. *Nat. Microbiol.* **4**, 2430–2441.
53. Scannell, D.R., Frank, A.C., Conant, G.C., Byrne, K.P., Woolfit, M., and Wolfe, K.H. (2007). Independent sorting-out of thousands of duplicated gene pairs in two yeast species descended from a whole-genome duplication. *Proc. Natl. Acad. Sci. USA* **104**, 8397–8402.
54. Bowers, J.E., Chapman, B.A., Rong, J., and Paterson, A.H. (2003). Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature* **422**, 433–438.
55. Paterson, A.H., Bowers, J.E., and Chapman, B.A. (2004). Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. *Proc. Natl. Acad. Sci. USA* **101**, 9903–9908.
56. Brunet, F.G., Roest Crolius, H., Paris, M., Aury, J.-M., Gibert, P., Jaillon, O., Laudet, V., and Robinson-Rechavi, M. (2006). Gene loss and evolutionary rates following whole-genome duplication in teleost fishes. *Mol. Biol. Evol.* **23**, 1808–1816.
57. Macdonald, D., Thomson, D.D., Johns, A., Contreras Valenzuela, A., Gilsenan, J.M., Lord, K.M., Bowyer, P., Denning, D.W., Read, N.D., and Bromley, M.J. (2018). Inducible cell fusion permits use of competitive fitness profiling in the human pathogenic fungus *Aspergillus fumigatus*. *Antimicrob. Agents Chemother.* **63**, e01615-18.
58. Olarte, R.A., Worthington, C.J., Horn, B.W., Moore, G.G., Singh, R., Monacell, J.T., Dorner, J.W., Stone, E.A., Xie, D.-Y., and Carbone, I. (2015). Enhanced diversity and aflatoxigenicity in interspecific hybrids of *Aspergillus flavus* and *Aspergillus parasiticus*. *Mol. Ecol.* **24**, 1889–1909.
59. Kevei, F., and Peberdy, J.F. (1984). Further studies on protoplast fusion and interspecific hybridization within the *Aspergillus nidulans* group. *J. Gen. Microbiol.* **130**, 2229–2236.
60. Kevei, F., and Peberdy, J.F. (1979). Induced segregation in interspecific hybrids of *Aspergillus nidulans* and *Aspergillus rugulosus* obtained by protoplast fusion. *Mol. Gen. Genet.* **170**, 213–218.
61. Menardo, F., Praz, C.R., Wyder, S., Ben-David, R., Bourras, S., Matsumae, H., McNally, K.E., Parlange, F., Riba, A., Roffler, S., et al. (2016). Hybridization of powdery mildew strains gives rise to pathogens on novel agricultural crop species. *Nat. Genet.* **48**, 201–205.
62. Zhou, X., Peris, D., Kominek, J., Kurtzman, C.P., Hittinger, C.T., and Rokas, A. (2016). *In silico* whole genome sequencer and analyzer (iWGS): a computational pipeline to guide the design and analysis of *de novo* genome sequencing studies. *G3* **6**, 3655–3662.

63. Stanke, M., and Waack, S. (2003). Gene prediction with a hidden Markov model and a new intron submodel. *Bioinformatics* 19 (Suppl 2), ii215–ii225.
64. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10, 421.
65. Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24, 1586–1591.
66. Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780.
67. Capella-Gutiérrez, S., Silla-Martínez, J.M., and Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25, 1972–1973.
68. Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313.
69. Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., and Minh, B.Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274.
70. Boeva, V., Popova, T., Bleakley, K., Chiche, P., Cappo, J., Schleiermacher, G., Janoueix-Lerosey, I., Delattre, O., and Barillot, E. (2012). Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. *Bioinformatics* 28, 423–425.
71. Weischenfeldt, J., and Porse, B. (2008). Bone marrow-derived macrophages (BMM): isolation and applications. *CSH Protoc.* 2008, pdb.prot5080.
72. Almeida, A.J., Matute, D.R., Carmona, J.A., Martins, M., Torres, I., McEwen, J.G., Restrepo, A., Leão, C., Ludovico, P., and Rodrigues, F. (2007). Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. *Fungal Genet. Biol.* 44, 25–31.
73. Sarkar, D., Le Meur, N., and Gentleman, R. (2008). Using flowViz to visualize flow cytometry data. *Bioinformatics* 24, 878–879.
74. Malavazi, I., and Goldman, G.H. (2012). Gene disruption in *Aspergillus fumigatus* using a PCR-based strategy and in vivo recombination in yeast. *Methods Mol. Biol.* 845, 99–118.
75. Mead, M.E., Raja, H.A., Steenwyk, J.L., Knowles, S.L., Oberlies, N.H., and Rokas, A. (2019). Draft genome sequence of the griseofulvin-producing fungus *Xylaria flabelliformis* strain G536. *Microbiol. Resour. Announc.* 8, e00890-19.
76. Sambrook, J., and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*, Third Edition (Cold Spring Harbor Laboratory).
77. Safonova, Y., Bankevich, A., and Pevzner, P.A. (2015). dipSPAdes: assembler for highly polymorphic diploid genomes. *J. Comput. Biol.* 22, 528–545.
78. Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Pribelski, A.D., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477.
79. Chikhi, R., and Medvedev, P. (2014). Informed and automated k-mer size selection for genome assembly. *Bioinformatics* 30, 31–37.
80. Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075.
81. Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H.U., Brucoleri, R., Lee, S.Y., Fischbach, M.A., Müller, R., Wohlleben, W., et al. (2015). antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* 43 (W1), W237–W243.
82. Ortiz-Merino, R.A., Kuanyshvov, N., Braun-Galleani, S., Byrne, K.P., Porro, D., Branduardi, P., and Wolfe, K.H. (2017). Evolutionary restoration of fertility in an interspecies hybrid yeast, by whole-genome duplication after a failed mating-type switch. *PLoS Biol.* 15, e2002128.
83. Madden, T. (2013). The BLAST sequence analysis tool. The NCBI Handbook, Second Edition (National Center for Biotechnology Information), pp. 1–17.
84. Mount, D.W. (2008). Using BLOSUM in sequence alignments. *CSH Protoc.* 2008, pdb.top39.
85. Suyama, M., Torrents, D., and Bork, P. (2006). PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Res.* 34, W609–12.
86. Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., and Zdobnov, E.M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31, 3210–3212.
87. Waterhouse, R.M., Tegenfeldt, F., Li, J., Zdobnov, E.M., and Kriventseva, E.V. (2013). OrthoDB: a hierarchical catalog of animal, fungal and bacterial orthologs. *Nucleic Acids Res.* 41, D358–D365.
88. Li, L., Stoeckert, C.J., Jr., and Roos, D.S. (2003). OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* 13, 2178–2189.
89. van Dongen, S. (2000). Graph clustering by flow simulation. PhD thesis (University of Utrecht).
90. Hoang, D.T., Chernomor, O., von Haeseler, A., Minh, B.Q., and Vinh, L.S. (2018). UFBoot2: improving the ultrafast bootstrap approximation. *Mol. Biol. Evol.* 35, 518–522.
91. Yang, Z. (1994). Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J. Mol. Evol.* 39, 306–314.
92. Yang, Z. (1996). Among-site rate variation and its impact on phylogenetic analyses. *Trends Ecol. Evol.* 11, 367–372.
93. Tavaré, S. (1986). Some probabilistic and statistical problems in the analysis of DNA sequences. *Lect. Math. Life Sci.* 17, 57–86.
94. Vinet, L., and Zhedanov, A. (2011). A ‘missing’ family of classical orthogonal polynomials. *J. Phys. A Math. Theor.* 44, 085201.
95. Abyzov, A., Urban, A.E., Snyder, M., and Gerstein, M. (2011). CNVnator: an approach to discover, genotype, and characterize typical and atypical CNVs from family and population genome sequencing. *Genome Res.* 21, 974–984.
96. Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv*, arXiv:1303.3997v2. <https://arxiv.org/abs/1303.3997>.
97. Wallace, D.P. (2004). The Mann-Whitney test. *J. Am. Soc. Inf. Sci. Technol.* 55, 93.
98. Panchenko, P. (2006). Kolmogorov-Smirnov test. *Stat. Appl.* 83–90.
99. Bom, V.L.P., de Castro, P.A., Winkelströter, L.K., Marine, M., Hori, J.I., Ramalho, L.N.Z., dos Reis, T.F., Goldman, M.H.S., Brown, N.A., Rajendran, R., et al. (2015). The *Aspergillus fumigatus* sitA phosphatase homologue is important for adhesion, cell wall integrity, biofilm formation, and virulence. *Eukaryot. Cell* 14, 728–744.
100. Drewniak, A., Gazendam, R.P., Tool, A.T.J., van Houdt, M., Jansen, M.H., van Hamme, J.L., van Leeuwen, E.M.M., Roos, D., Scalais, E., de Beaufort, C., et al. (2013). Invasive fungal infection and impaired neutrophil killing in human CARD9 deficiency. *Blood* 121, 2385–2392.
101. Gazendam, R.P., van Hamme, J.L., Tool, A.T.J., Hoogenboezem, M., van den Berg, J.M., Prins, J.M., Vitkov, L., van de Veerdonk, F.L., van den Berg, T.K., Roos, D., and Kuijpers, T.W. (2016). Human neutrophils use different mechanisms to kill *Aspergillus fumigatus* conidia and hyphae: evidence from phagocyte defects. *J. Immunol.* 196, 1272–1283.
102. Dos Reis Almeida, F.B., Carvalho, F.C., Mariano, V.S., Alegre, A.C., Silva, Rdo.N., Hanna, E.S., and Roque-Barreira, M.C. (2011). Influence of N-glycosylation on the morphogenesis and growth of *Paracoccidioides brasiliensis* and on the biological activities of yeast proteins. *PLoS ONE* 6, e29216.
103. Lessing, F., Knemeyer, O., Wozniak, I., Loeffler, J., Kurzai, O., Haertl, A., and Brakhage, A.A. (2007). The *Aspergillus fumigatus* transcriptional regulator AfYap1 represents the major regulator for defense against

reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. *Eukaryot. Cell* 6, 2290–2302.

104. Lambou, K., Lamarre, C., Beau, R., Dufour, N., and Latge, J.-P. (2010). Functional analysis of the superoxide dismutase family in *Aspergillus fumigatus*. *Mol. Microbiol.* 75, 910–923.
105. Warris, A., and Ballou, E.R. (2019). Oxidative responses and fungal infection biology. *Semin. Cell Dev. Biol.* 89, 34–46.
106. Kassambara, A., and Mundt, F. (2017). factoextra. R Packag. v. 1.0.5.
107. Lê, S., Josse, J., and Husson, F. (2008). FactoMineR : an R package for multivariate analysis. *J. Stat. Softw.* 25, 1–18.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
<i>A. spinulosporus</i> NRRL 2395 ^T	[30]; USDA ARS Culture Collection Database	Strain # NRRL 2395 ^T
<i>A. nidulans</i> A4	[36]; Fungal Genetics Stock Center	Strain # A4
<i>A. quadrilineatus</i> NRRL 201 ^T	This study; USDA ARS Culture Collection Database	Strain # NRRL 201 ^T
<i>A. spinulosporus</i> 4060	[28]; Laboratory of Dr. Donald Sheppard, McGill University	Strain # 4060
<i>A. latus</i> NRRL 200 ^T	This study; USDA ARS Culture Collection Database	Strain # NRRL 200 ^T
<i>A. latus</i> MM151978	This study; Laboratory of Dr. Donald Sheppard, McGill University	Strain # MM151978
<i>A. latus</i> NIH	This study; Clinical Laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven	Strain # NIH
<i>A. latus</i> ASFU1710	This study; Clinical Laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven	Strain # ASFU1710
<i>A. latus</i> ASFU1908	This study	N/A
<i>A. latus</i> ASFU2033	This study; Clinical Laboratory of Dr. Katrien Lagrou, Department of Microbiology, Immunology and Transplantation, KU Leuven	Strain # ASFU2033
<i>A. latus</i> MO46149	This study; Clinical Laboratory of Dr. Catarina Lameiras, Department of Microbiology, Portuguese Oncology Institute of Porto	Strain # MO46149
Macrophages	This study	N/A
Neutrophils	This study	N/A
Human polymorphonuclear cells	This study	N/A
Human blood samples	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
Caspofungin	Sigma-Aldrich	Cat#SML0425
Amphotericin B	Sigma-Aldrich	Cat#A2942
Itraconazole	Sigma-Aldrich	Cat#I6657
Voriconazole	Sigma-Aldrich	Cat#PZ0005
Posaconazole	Sigma-Aldrich	Cat#P-103
Menadione	Sigma-Aldrich	Cat#M5625
Hydrogen peroxide	Sigma-Aldrich	Cat#H3410
Paraquat	Sigma-Aldrich	Cat#36541
Bathophenanthrolinedisulfonic acid (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid [BPS])	Sigma-Aldrich	Cat#B1375
3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine)	Sigma-Aldrich	Cat#P9762

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium- 5-carboxanilide sodium salt))	Sigma-Aldrich	Cat#X4626
MTT	Sigma-Aldrich	Cat#298-93-1
PMA (phorbol 12-myristate 13-acetate)	Sigma-Aldrich	Cat#16561-29-8
LIVE/DEAD	Invitrogen	Cat#R37601
SYTOX Green Nucleic Acid Stain	Invitrogen	Cat#S7020
Percoll	GE Healthcare	Cat#17-5445-02
Proteinase K	Sigma-Aldrich	Cat#39450-01-6
SYBR Green 10,000x	Thermo Fisher Scientific Inc.	Cat#S7567
Triton® X-100	Sigma-Aldrich	Cat#9002-93-1
Deposited Data		
Raw reads used for genomics; See Table S3	This study	N/A
Genome assemblies; See Table S3	This study	N/A
Predicted gene boundaries	This study	figshare: 10.6084/m9.figshare.8114114
Phenotypic measurements	This study	figshare: 10.6084/m9.figshare.8114114
FACS data	This study	figshare: 10.6084/m9.figshare.8114114
Phylogenetic and phylogenomic data matrices	This study	figshare: 10.6084/m9.figshare.8114114
Parent of origin analysis	This study	figshare: 10.6084/m9.figshare.8114114
Homeologs per hybrid isolates	This study	figshare: 10.6084/m9.figshare.8114114
Biosynthetic gene cluster predictions	This study	figshare: 10.6084/m9.figshare.8114114
Experimental Models: Organisms/Strains		
The greater wax moth (<i>Galleria mellonella</i>)	Goldman lab	N/A
Software and Algorithms		
<i>In silico</i> Whole Genome Sequencer and Analyzer (iWGS), version 1.1	[62]	https://github.com/zhouxiaofan1983/iWGS
AUGUSTUS, version 3.3	[63]	http://bioinf.uni-greifswald.de/augustus/
Blast+, version 2.3.0	[64]	https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+
PAML, version 4.9	[65]	http://abacus.gene.ucl.ac.uk/software/paml.html
Mafft, version 7.402	[66]	https://mafft.cbrc.jp/alignment/software/
trimAl, version 1.2rev59	[67]	http://trimal.cgenomics.org/downloads
RAxML, version 8.2.11	[68]	https://cme.h-its.org/exelixis/web/software/raxml/
IQTREE, version 1.6.1	[69]	http://www.iqtree.org
Control-FREEC, version 9.1	[70]	https://github.com/BoevaLab/FREEC

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Antonis Rokas (antonis.rokas@vanderbilt.edu).

Materials Availability

This study did not generate new unique reagents. *Aspergillus* strains are available upon request.

Data and Code Availability

All data are publicly available through NCBI or a figshare repository. Genome assemblies are available through BioProject IDs PRJNA542678 and PRJNA542141; raw reads are available through BioProject IDs PRJNA542395, PRJNA542181, and PRJNA542141. Genome assembly and raw reads for *A. quadrilineatus* NRRL 201^T are available through BioProject ID PRJNA623402. Strain specific BioProject or BioSample IDs can be found in Table S3. The figshare repository (<https://doi.org/10.6084/m9.figshare.8114114>) contains phenotypic measurements, parent of origin analysis, homeologs per hybrid isolate, fluorescence-assisted cell sorting files, phylogenetic and phylogenomic data matrices and tree files, biosynthetic gene cluster prediction results, genome assemblies and gene predictions. Additional scripts and commands used throughout computational analyses are available through GitHub https://github.com/JLSteenwyk/Pathogenic_allodiploid_hybrids_of_AspERGillius_fungi.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Microbe strains

All *Aspergillus* spp. strains were obtained from public collections, in laboratories, or hospitals. Upon receipt, these strains were grown on YAG medium and after 5 days growth at 30°C, and asexual spores (conidia) were harvested and stored either at 4°C in water for further usage or at –80°C in a 30% glycerol solution in the laboratory collection. In experiments for DNA extraction and genome sequencing, the conidia were inoculated into Erlenmeyer-flasks on an orbital shaker (150 rpm) at 30°C for 24 h. *A. quadrilineatus* NRRL 201^T was acquired separately from the NRRL culture collection. Upon receipt of the cryopreserved vial, the contents of the frozen material were aseptically removed from the vial and transferred to 50 mL of YESD (Yeast Extract, Soy peptone, Dextrose) media. After, 3–4 days of shaking on an orbital shaker (100 rpm) at room temperature, the resulting mycelium was aseptically transferred on potato dextrose agar for all subsequent experiments, including DNA extraction and genome sequencing. Cultures of *A. quadrilineatus* NRRL201^T are currently preserved at room temperature on potato dextrose agar (Difco).

Primary cell cultures

To obtain macrophages for a phagocytosis assay of *Aspergillus* asexual spores (conidia), we used bone marrow-derived macrophages that were isolated as described previously [71]. Briefly, macrophages were recovered from femurs of male C57BL/6 (wild-type, WT, Jax 000664) at 6–8 weeks old and were incubated in an RPMI medium (GIBCO) supplemented with 30% (volume / volume) L929 growth conditioning media, 20% inactivated fetal bovine serum (GIBCO), 2 mM glutamine and 100 units / milliliter of penicillin-streptomycin (Life Technologies). Fresh media was added after 4 days of cultivation and macrophages were collected after 7 days and used for subsequent experiments. The animals were housed in the animal facility of the Ribeirao Preto Medical School, University of Sao Paulo, under optimized hygienic conditions.

Human polymorphonuclear cells (PMNs) were isolated from 8 mL of peripheral blood of adult male healthy volunteers by density centrifugation using Mono-Poly Resolving Medium (MP Biomedicals LLC, Irvine, CA, USA) according to the manufacturer's instruction. The PMNs (5 × 10⁶ / mL) were resuspended in Hank's Balanced Salt Solution, without calcium or magnesium, containing 5% fetal bovine serum (GIBCO®, South American, Brazil).

Animals

Galleria mellonella larvae are cultivated in a greenhouse at 27°C. The larvae are kept in glass jars, in which they are separated by stages, moths, eggs, larvae and pupa.

The larvae are fed once a week, with a feed composed of (yeast extract 1.5%; soy 1%, powdered milk 1%, honey 1% and beeswax 2%).

In the sixth week of the larval stage, the larvae are adult and larger, facilitating infection.

We separated and assembled the groups with the larvae (n = 10) in Petri dishes. The groups are composed of larvae that are approximately 300 mg in weight and 2 cm long. Moth sex was not accounted for due to the impossibility of visually determining sex at the sixth week of larval development.

Ethics statement

The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO on the 27th January 1978 in its 8th and 14th articles. All protocols used in this study were approved by the local ethics committee for animal experiments from Universidade de São Paulo, Campus Ribeirão Preto (permit number 08.1.1277.53.6). All animals were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation (Princípios Éticos na Experimentação Animal—Colégio Brasileiro de Experimentação Animal [COBEA]) and the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

METHOD DETAILS

Fluorescence-assisted cell sorting for DNA content determination

Asexual spores (conidia) were collected, centrifuged (13,000 rounds per minute for 3 min) and washed with sterile 1 x phosphate-buffered saline (8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium phosphate, 0.24 g monopotassium phosphate

per liter of sterilized water). For cell staining, the protocol described by Almeida et al. [72] was followed with modifications. Following overnight fixation with 70% ethanol (volume / volume) at 4°C, spores were harvested, washed and suspended in 850 μ l of sodium citrate buffer (50 mM sodium citrate; pH = 7.5). Spores were subsequently sonicated using four ultrasound pulses at 40W for 2 s with an interval of 1 to 2 s between pulses. Sonicated spores were treated for 1 h at 50°C with RNase A (0.50 mg / milliliter; Invitrogen, Waltham, Massachusetts, USA) and for 2 h at 50°C with proteinase K (1 mg/mL; Sigma-Aldrich, St. Louis, Missouri, USA). Spores were stained overnight with SYBR Green 10,000x (Invitrogen, Carlsbad, CA, USA) diluted 10-fold in Tris-ethylenediaminetetraacetic acid (pH 8.0), at a concentration of 2% (volume / volume) at 4°C. Finally, Triton® X-100 (Sigma-Aldrich) was added to samples at a final concentration of 0.25% (volume / volume). Stained spores were analyzed in a Fluorescence-assisted cell sorting LSRII flow cytometer (Becton Dickinson, NJ, USA) with a 488 nanometer excitation laser. Signals from a minimum of 30,000 cells per sample were captured in fluorescein isothiocyanate channel (530 nanometers $\pm$ 30 nanometers) at low flow rate of $\sim$ 1,000 cells / second and an acquisition protocol was defined to measure forward scatter and side scatter on a four-decade logarithmic scale and green fluorescence on a linear scale. Fluorescence-assisted cell sorting Diva was used as the acquisition software. Results were analyzed with the R package flowViz, version 1.46.1 [73].

Asexual spore size measurements

The diameters of 100 spores for each isolate were measured under a Carl Zeiss (Jena, Germany) AxioObserver.Z1 fluorescent microscope equipped with a 100-W HBO mercury lamp using the 100 x magnification oil immersion objective and the AxioVision, software v.3.1.

DNA extraction and sequencing

Frozen mycelia of all isolates were ground in liquid nitrogen and genomic DNA was extracted as previously described [74, 75]. Standard techniques for manipulation of DNA were used [76]. The genomes of all clinical isolates and the type strain of *A. latus* (9 in total) were sequenced at the Genomic Services Lab of Hudson Alpha (Huntsville, Alabama, USA) on an Illumina HiSeq 2500 sequencer; the sole exception was *A. quadrilineatus* NRRL 201^T, which was sequenced using on a NovaSeq S4 at the Vanderbilt Technologies for Advanced Genomes facility (Nashville, Tennessee, USA). All isolates were sequenced using paired-end sequencing (150 bp) with the Illumina TruSeq library kit. Additionally, the type strain of *A. latus* and the clinical isolates MM151978 and NIH were also sequenced using mate-pair sequencing (150 bp) using the Illumina Nextera Mate Pair Library kit with an insert size of 4 kilobases. The genome coverage of each isolate was greater than 150X. Both the raw short-read sequence data and the genome assemblies are publicly available (see Table S3 for accession numbers).

Genome assembly and annotation

All genomes were assembled with the iWGS pipeline [62] using dipSPAdes, version 1.0 [77] or SPAdes, version 3.6.2 [78]. Optimal *k*-mer lengths were selected using KmerGenie, version 1.6982 [79], and assembly quality was evaluated using QUAST, version 3.2 [80]. Protein-coding genes were predicted using AUGUSTUS, version 3.3 [63], using *Aspergillus nidulans* gene annotation parameters. Predicted genes in each *A. latus* hybrid genome were annotated by reciprocal-best-BLAST against a database of all *A. nidulans* and *A. spinulosporus* proteins.

Prediction of secondary metabolic gene clusters

Secondary metabolic gene clusters (SMGCs) were predicted in all assembled genomes using antiSMASH, version 3.0.5.1 [81]. In addition, we used literature-curated SMGC annotations from the well-characterized *A. nidulans* A4 genome [36] to identify SMGCs not captured by the antiSMASH software.

Assigning genes in hybrid genomes to parents of origin

To determine the most likely parent-of-origin for each gene in a hybrid genome, we measured the sequence divergence between every gene in a hybrid genome and its ortholog in the *A. spinulosporus* NRRL 2395^T parent [82]. Specifically, for each gene in a hybrid genome, we used BlastP, version 2.3.0 from NCBI's Blast+ package [83], to identify the putative *A. spinulosporus* ortholog. The resulting pair was then aligned using Mafft, version 7.294b [66], with the Blosum 62 matrix of substitutions [84], a gap penalty of 1.0, 1,000 maximum iterations, a single guide tree, and the 'genafpair' parameter. The associated DNA sequences were then forced onto the protein alignment using PAL2NAL, version 14 [85], and the synonymous substitution rate *K*_s was calculated according to LWL85m method using yn00 module in paml, version 4.9 [65]. By examining the bimodal distribution of *K*_s values, genes with relatively low *K*_s values ($0 \leq K_s < 0.05$) were inferred to be from the *A. spinulosporus* parent genome and genes with high *K*_s values ($0.05 \leq K_s < 10$) were inferred to stem from the *A. quadrilineatus*-like parent genome. We performed the same analysis on the known, non-hybrid haploid genome of *Aspergillus fumigatus* strain A1163 compared to *A. fumigatus* strain Af293 [33] and on the known hybrid diploid genome of *ZygoSaccharomyces parabailii* strain NBRC1047/ATCC56075 compared to their closest homologs in the known parent *ZygoSaccharomyces bailii* strain CLIB213 [82] as controls.

To determine the completeness of each parental genome and evaluate the efficacy of assigning genes in hybrid genomes to parent-of-origin, we created separate proteome FASTA files for genes from the *A. spinulosporus* and *A. quadrilineatus*-like regions

of the genome and evaluated how many near-universally single copy orthologous genes were present in each parental genome using the BUSCO pipeline [86] with the Ascomycota database (creation date: 2016-02-13, number of species: 75, number of BUSCOs: 1315) from OrthoDB, version 9 [87].

To determine homeolog pairs between each parental genome, we used a reciprocal best blast hit approach. Specifically, we used the FASTA files of genes created in the previous step and conducted a reciprocal best blast hit analysis between the two parental genomes using an e-value cutoff of 10^{-3} . To determine if one gene was putatively pseudogenized, we employed a previously established approach, which compares gene lengths between the two homeologs; genes whose length is substantially shorter (we used an upper threshold of 80%) than their homeolog pair are inferred to be pseudogenes [82].

Maximum likelihood phylogenetic and phylogenomic analyses

To establish the taxonomic identity of the sequenced isolates, we retrieved their β -tubulin and calmodulin sequences by performing a nucleotide BLAST of the *Aspergillus nidulans* A4 β -tubulin and calmodulin sequences against each assembled genome. In addition, β -tubulin and calmodulin sequences from two strains of each of *Aspergillus foveolatus*, *Aspergillus latus*, *Aspergillus nidulans*, *Aspergillus pachycristatus*, *Aspergillus rugulosus*, *Aspergillus spinulosporus*, and *Aspergillus striatus* and from one strain of *Aspergillus corrugatus* were retrieved from GenBank. We also retrieved the same sequences from one strain of *Aspergillus sydowii*, which served as an outgroup for the phylogeny [37]. Sequences were aligned with MAFFT, version 7.310 [66], gaps were removed with trimAl, version 1.2.rev59 [67], using the 'gappypout' parameter, and the β -tubulin and calmodulin sequences for each individual strain were concatenated. A phylogeny was constructed from the concatenated sequences with RAxML, version 8.2.11 [68], with the GTRGAMMAX model and 1,000 rapid bootstrap replicates. Branches with bootstrap support less than 80 were collapsed.

To determine the number of hybridization events that gave rise to *A. latus* isolates, we conducted phylogenomic analyses to reconstruct the evolutionary history of the *A. spinulosporus* parental genome in the *A. latus* hybrids and of the genes of *A. spinulosporus* strains NRRL2395 and 4060. We first identified single-copy orthologous genes across the 9 strains using OrthoFinder, version 2.3.8 [88] with default parameters, which employs a Markov clustering algorithm [89] on gene sequence similarity information derived from an 'all-versus-all' approach using NCBI's Blast+, version 2.3.0. Out of the inferred 12,596 groups of orthologous genes, 5,894 were single-copy (i.e., each of the 9 isolates were represented by a single sequence). All 5,894 sets of corresponding nucleotide sequences were aligned using MAFFT, version 7.402 [66], with the 'genafpair' parameter and 1,000 maximum iterative sequence alignment refinements. Alignments were trimmed using trimAl, version 1.2.rev59 [67], using the 'gappypout' parameter. The resulting sequences were concatenated into a single nucleotide data matrix (8,405,004 sites). The strain phylogeny was inferred using iqtree, version 1.6.1 [69], with the nbest parameter set to 10 to increase the number of best trees used during the search. Bipartition support was evaluated using 5,000 ultrafast bootstrap approximation replicates [90]. The evolutionary history of the *A. quadrilineatus*-like parental genome among the *A. latus* hybrids and two *A. quadrilineatus* strains (NRRL201^T and CBS 853.96) was inferred using the same approach; in this case, the nucleotide data matrix was comprised of 7,385,465 sites (from 5,079 single-copy orthologous genes out of a total of 11,814 groups of orthologous genes).

Lastly, we conducted approximately unbiased topology tests [38] using the data matrices from each parental genome with the 'au' parameter in iqtree, version 1.6.1 [69]. Specifically, we examined if the two topologies among the 7 *A. latus* hybrids inferred using the *A. spinulosporus* or *A. quadrilineatus*-like parental genomes were significantly different for either genome-scale data matrix inferred from the parental genomes. During constrained tree search, we used a substitution model of a general time-reversible model with empirical base frequencies, a discrete Gamma model with 4 rate categories, and allowed for a proportion of invariable sites (GTR+I+F+G4) [91–94].

Examination of loss of heterozygosity using copy number variation analysis

To conservatively and accurately instances of loss of heterozygosity, we measured copy number variation using Control-FREEC, version 9.1 [70] and CNVnator, version 0.3.2 [95]. More specifically, we evaluated false discovery rate (FDR) and false positive rate (FPR) using reads from *A. spinulosporus* that were aligned to a "concatenated" genome of the *A. spinulosporus* and *A. nidulans* FGSC-A4 using 10 different window sizes (500, 750, 1000, 1250, 1500, 1750, 2000, 3000, 4000, and 5000 base pairs). Reads were aligned with bwa-mem, version 0.7.12 [96], and duplicates reads were removed with picardtools, version 2.1.1 (<http://broadinstitute.github.io/picard/>). In this way, we inferred the window size parameter that minimizes the FDR and FPR CN variable regions identified in the hybrid genomes. Specific parameters used for Control-FREEC include a minimum and maximum expected GC-content of 0.3 and 0.5, respectively, and a telocentrometric parameter of 10,000. Default parameters were used for CNVnator. To identify statistically significant CN variable loci, we implemented a Wilcoxon Rank Sum test [97] and a Kolmogorov-Smirnov test [98], in the case of Control-FREEC and a t test in the case of CNVnator. Using the resulting set of significant CN variants per window size for each program, FDR and FPR were calculated using the following formulas:

$$FDR = 1 - TP / (TP + FP)$$

$$FPR = FP / (FP + TN)$$

where *TP* represents true positives, *FP* represents false positives, and *TN* represents true negatives. Across the 10 different window sizes, we found that Control-FREEC often, but not always, slightly outperformed CNVnator (figshare image 5, 10.6084/m9.figshare.8114114S5S10). More importantly, we found that Control-FREEC had an FDR and FPR of 0 (i.e., had no false negatives or false positives) when using a window size of 1000 and 1250. Therefore, we used Control-FREEC with a window size of 1000 to identify CN variable loci in all other isolates.

Macrophage isolation

To obtain macrophages for a phagocytosis assay of *Aspergillus* asexual spores (conidia), we used bone marrow-derived macrophages that were isolated as described previously [71]. Briefly, macrophages were recovered from femurs of C57BL/6 wild-type mice (6-weeks old) and were incubated in an RPMI medium (GIBCO) supplemented with 30% (volume / volume) L929 growth conditioning media, 20% inactivated fetal bovine serum (GIBCO), 2 mM glutamine and 100 units / milliliter of penicillin-streptomycin (Life Technologies). Fresh media was added after 4 days of cultivation and macrophages were collected after 7 days and used for subsequent experiments.

In vitro phagocytosis by macrophages

Phagocytosis of asexual spores (conidia) by wild-type macrophages were carried out according as previously described with modifications [99]. 24-well plates containing a 15-mm-diameter coverslip in each well (phagocytosis assay) and 2×10^5 macrophages per well were incubated with 1 mL of RPMI-FBS [(RPMI medium (GIBCO) supplemented with 10% inactivated fetal bovine serum (GIBCO), 2 mM glutamine and 100 units / milliliter of penicillin-streptomycin (Life Technologies)] at 37°C, 5% carbon dioxide for 24 h. Wells were washed with 1 mL of phosphate-buffered saline before the same volume of RPMI-FBS medium supplemented with 1×10^6 spores (1:5 macrophage / spore ratio) was added in the same conditions.

To determine phagocytosis, macrophages were incubated with spores for 1.5 h before supernatant was removed and 500 μ L of phosphate-buffered saline containing 3.7% formaldehyde was added for 15 min at room temperature. Sample coverslips were washed with 1 mL of ultrapure water and incubated for 20 min with 500 μ L of 0.1 mg / milliliter calcofluor white to stain the cell wall of non-phagocytosed spores. Samples were washed and coverslips were viewed under a Zeiss Observer Z1 fluorescence microscope. In total, 100 spores were counted per sample and the phagocytosis index was calculated. Experiments were performed in biological triplicates.

Viability of *Aspergillus* hyphae

Human neutrophils from fresh venous blood of healthy adult volunteers were isolated according to a previous study with slight modifications [100], through centrifugation over isotonic Percoll, lysed, and resuspended in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline solution. Since we did not observe any neutrophil-mediated killing of *Aspergillus* asexual spores, as also previously described [101], we followed the protocol previously reported for neutrophil-mediated inhibition of germination. *Aspergillus* asexual spores were incubated with neutrophils (0.5, 1.0, or 2.5×10^5 cells / milliliter; effector: target cell ratios of 1:1000, 1:500, or 1:200, respectively) in a 96-well plate overnight at 37°C in RPMI 1640 medium containing glutamine and 10% fetal calf serum (Life). The neutrophils were lysed in water / sodium hydroxide (pH 11.0) and spore germination was determined using an MTT (thiazolyl blue; Sigma-Aldrich) assay as previously reported [102]. Each isolate's viability was calculated relative to incubation without neutrophils, which was set at 100% for each isolate and evaluated separately. To evaluate the viability of *Aspergillus* hyphae in the presence of neutrophils, we used a previously described protocol [101]. *Aspergillus* asexual spores were incubated overnight at 37°C in RPMI 1640 medium containing glutamine and 10% fetal calf serum (Life) upon formation of a monolayer, as verified by microscope. Freshly isolated neutrophils (0, 1.0, 2.0, or 3.0×10^5 cells / milliliter) were cultured for 1 h on the *Aspergillus* monolayer at 37°C. Subsequently, the cells were lysed in water / sodium hydroxide (pH 11.0) and the MTT assay was performed. Each isolate's hyphal viability was calculated as a percentage of its viability after incubation without neutrophils. The experiments were repeated three times, each performed in triplicate.

NETosis assays

Human polymorphonuclear cells (PMN) were isolated from 8 mL of peripheral blood of adult male healthy volunteers by density centrifugation using Mono-Poly Resolving Medium (MP Biomedicals LLC, Irvine, CA, USA) according to the manufacturer's instruction. PMN (5×10^6 / mL) were resuspended in Hank's Balanced Salt Solution, without calcium or magnesium, containing 5% FBS (GIBCO®, South American, Brazil). For flow cytometry analysis, 100 μ L (5×10^5 / tube) of PMN were seeded in sterile round-bottom polystyrene tubes, stimulated with 10 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) or fungi samples (5×10^5 / tube) and then incubated for 3 h at 37°C and 5% CO₂. In the last 30 min, 1000x diluted LIVE/DEAD (Invitrogen, Eugene, OR, USA) was added. After that, PMN were made to react with SYTOX Green Nucleic Acid Stain (1 μ M) (Invitrogen) for 10 min at room temperature. Data on cells were acquired by flow cytometry using a BD FACSCanto II instrument (BD Bioscience, Franklin Lakes, NJ, USA). One hundred thousand events per sample were collected, doublet discrimination was performed using Forward Scatter Area (FSC-A) versus Forward Scatter Height (FSC-H) parameters, and the PMN were gated according to size (FSC-A) and granularity (Side Scatter Area, SSC-A). LIVE/DEAD and SYTOX positive cells were analyzed with FlowJo software (TreeStar, Ashland, OR, USA). For fluorescence microscopy, 100 μ L (5×10^5 / well) of PMN were seeded on 13 mm glass coverslips in 24-wells plate and pre-incubated for 30 min at 37°C and 5% CO₂. After adherence, PMN were stimulated with PMA (10 nM) or fungi samples

(5×10^5 / tube) and then incubated for 3 h at 37°C and 5% CO₂. PMN were made to react with SYTOX Green (1 μ M) (Life Technologies) for 10 min at room temperature. The glass coverslips were removed and the slides were fixed with ProLong Gold Antifade Mountant with DAPI (Invitrogen). The images were obtained using a Leica DMI6000 Fluorescence Microscope (Leica Microsystems, Wetzlar, Germany). Details on the flow cytometry gating strategy used and microscopy images are available in the figshare repository (<https://doi.org/10.6084/m9.figshare.8114114>).

Growth in the presence of different stresses

To study variation in infection-relevant phenotypes between the hybrid isolates, we compared the radial growth of *A. nidulans*, *A. spinulosporus*, *A. latus*, and of all the clinical isolates in different temperatures (30°C, 37°C and 44°C), in the presence of increasing concentrations of oxidative stress-inducing compounds (paraquat and menadione), and on iron starvation. Although the importance of oxidative stress susceptibility is contentious for *Aspergillus* virulence [103, 104], we chose to examine these phenotypes because it is well established that hosts produce reactive oxygen species in response to infection [105]. To test for the effects of iron starvation on fungal growth, MM was prepared without any iron source and supplemented or not with 200 μ M of the iron chelators Bathophenanthrolinedisulfonic acid (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid [BPS]) (Sigma-Aldrich) and 300 μ M of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine) (Sigma-Aldrich). For radial growth, isolates were grown in triplicate from 10^5 spores and incubated at 37°C (except for the temperature test) for 5 days. Growth results in the presence of oxidative stress were expressed as ratio, dividing colony radial diameter (cm) of growth in the stress condition by colony radial diameter in the control (no stress condition).

Hydrogen peroxide tolerance

To test the viability of *Aspergillus* hyphae after exposure to hydrogen peroxide, we performed the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide sodium salt) (Sigma-Aldrich) assay as described by Henriot et al. [24], but with modifications. We obtained hyphae from each strain by incubating 1×10^5 asexual spores/well in 96-well plates containing MM. After 16 h at 37°C, the medium was removed and the wells washed twice with PBS. Subsequently, 100 μ l of minimal media supplemented or not (control) with different concentrations of hydrogen peroxide (1, 3 and 5 mM) were added to each well. Hyphal viability was assayed after 90 min of incubation (37°C) to avoid overgrowth of hyphae. One hundred microliters of PBS and 100 μ l of XTT-menadione solution was added to each well, obtaining a final concentration of 200 μ g / milliliter XTT and 4.3 μ g / milliliter menadione and the plates were incubated for 2 h in the dark. After centrifugation (3,000 $\times$ g for 10 min), the supernatants were transferred to another plate and read at 450 nm in a spectrophotometer. Fungal damage was defined as the percent reduction in metabolic activity compared to that of controls without hydrogen peroxide (viability = 100%).

Antifungal susceptibility assays

Antifungal susceptibility testing for voriconazole (Sigma-Aldrich), posaconazole (Sigma-Aldrich), itraconazole (Sigma-Aldrich) and amphotericin B was performed by determining the minimal inhibitory concentration (MIC) according to the protocol established by the Clinical and Laboratory Standards Institute (CLSI, 2017). For caspofungin (Sigma-Aldrich) susceptibility, the radial growth in MM supplemented with different concentration of the drug was carried out similarly as described before (see [Growth in the presence of different stresses](#)). The results were presented as a ratio: growth in caspofungin(cm)/growth in the control/without caspofungin (cm).

Lastly, to determine the extent of phenotypic differences across all strains tested and all traits measured, we conducted principal component analysis. To do so, we first scaled (i.e., standardized) the data to account for variables that are measured in different scales (e.g., MIC, radial growth, virulence). We then conducted principal component analysis and examined each variable's contribution to the variance along principal components using the R, version 3.5.2, packages factoextra, version 1.0.5 [106], and FactoMineR, version 1.41 [107].

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed in R, version 3.5.2 (<https://www.r-project.org/>). Throughout the manuscript, comparisons were made between *A. spinulosporus*, *A. quadrilineatus*, *A. latus*, and/or *A. nidulans* (e.g., [Figures 1, 3, and S5](#)). For these comparisons, we used a multi-factor ANOVA. If the null hypothesis was rejected, we used a Tukey's honest significant differences test to examine pairwise comparisons. To visualize these comparisons, we used violin plots with embedded boxplots ([Figure 1B](#)) or bar graphs ([Figures 3 and S5](#)). Violin plots depict the distribution of the underlying data; boxplot hinges depict the first, second, and third quartiles and whiskers extend to the largest value no greater than 1.5 times the interquartile range from the first or third quartile hinge; bar graphs depict the mean value measured across replicates and error bars depict the standard deviation across replicates. To conduct the principal component analysis (PCA) shown in [Figure 1A](#), we first scaled all the data. To examine heterogeneity in *Galleria mellonella* killing curves, we employed a log-rank test. To compare *Galleria mellonella* killing curves solely across *Aspergillus* strains, we removed the negative control (PBS) prior to conducting statistical analysis. Other statistical analyses were automatically performed by publicly available software (e.g., substitution model testing for phylogenomic analyses was automatically performed by IQ-TREE).

ANEXO E

Manuscrito: REZENDE, C. A.; BRITO, P. K. M. O.; PESSONI, A. M.; DA SILVA, T. A.; GOLDMAN, G. H; ALMEIDA, F. Altered expression of genes related to innate antifungal immunity in the absence of galectin-3. *Virulence*. v.12, p. 981-988, 2021.

RESEARCH PAPER

Altered expression of genes related to innate antifungal immunity in the absence of galectin-3

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ABSTRACT

Galectin-3 (Gal-3) is the most studied member of the animal galectin family, which comprises β -galactoside-binding lectins and participates in several cellular events. Its expression in cells involved in innate and adaptive immunity is related to anti- and proinflammatory functions, signaling an important role in inflammatory, infectious, and tumorigenesis processes. Mice deficient in Gal-3 exhibit important phenotypes, but it is unclear whether these phenotypes reflect an impairment of the functions of this protein. Gal-3 plays an important role in modulating the immune response to different pathogenic microorganisms. However, the role of Gal-3 in immunity to infection is still poorly understood. Therefore, we investigated the effects of Gal-3 deletion on the expression of genes involved in the innate immune response in the lungs, spleens, and brains of Gal-3 KO mice. Gene profiling expression analysis suggested that Gal-3 deletion resulted in differentially modulated expression of the genes encoding beta-glucan, mannose and chitin-responsive pattern recognition receptors, signal transduction, inflammation, and phagocytosis. Our data thus suggest the importance of Gal-3 expression in the host innate immune system.

ARTICLE HISTORY

Received 9 June 2020

Revised 19 February 2021

Accepted 5 March 2021

KEYWORDS

Galectin-3; gene knockout; innate immune response; antifungal immunity; gene expression

Introduction

Galectins are a family of animal β -galactoside-binding lectins [1,2]. This family consists of 15 multifunctional members [3], which are expressed in the nucleus, cytoplasm or cell membranes, or secreted extracellularly [1]. Galectins can bind glycoconjugates on the cell surface, triggering a transmembrane signaling cascade [3–5], which modulates several biological processes such as apoptosis, immune cell activation, cell adhesion, and cytokine secretion [6].

Galectin-3 (Gal-3) is the most studied member of the galectin family. The protein is expressed in various types of immune cells, tissues, and organs at different stages of development [7,8]. Its expression depends on the stage of the cell cycle and the metabolic status of the cell [9,10], while its biological functions are defined by its intracellular selectivity or extracellular localization [11]. Gal-3 can associate with the cell surface or localize in the extracellular matrix [12,13]. Extracellularly, Gal-3 exerts several autocrine and paracrine effects, which can mediate cell adhesion and activation by acting as chemoattractants for certain types of cells [3].

The expression of Gal-3 in cells of the innate and adaptive immune systems is related to anti- and proinflammatory functions [14–17], signaling important roles in inflammatory and infectious processes [16]. Depending on their ligands, Gal-3 has varied functions and participates in several cellular events [16]. Therefore, Gal-3 pathophysiological activities are associated with autoimmune diseases, tumorigenesis, and fungal or parasitic infections [18].

Notably, mice deficient in Gal-3 exhibit several important phenotypes such as impaired development [19], inflammatory functions [20] and stress control increased compulsive behavior [21] and high susceptibility to fungal infections [22–25]. Although it is unclear whether these phenotypes reflect an impairment of the intra- or extracellular functions of this protein [26]. In the population, the existence of single nucleotide polymorphisms (SNPs) in the gene encoding Gal-3 (*Igals3*) promotes variations in their serum levels in children with respiratory tract infections [27] and in diseases such as rheumatoid arthritis [28], malignant tumors [29], cardiomyopathies [30], but there is

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Supplemental data for this article can be accessed [here](#)

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nothing related to fungal infections. Studies show that SNPs in the *ptx3* [31] and *clec7a* [32] are associated with an increased risk of developing aspergillosis and candidiasis. Also, the presence of SNPs in host immunity genes, especially in standard recognition receptors (PRRs) such as toll-like receptors [33], C-type lectin receptors [34,35], pentraxins [36] and tumor necrosis factor receptors [37,38], may contribute to a greater susceptibility to fungal infections [39].

Therefore, we investigated whether Gal-3 deficiency promoted changes in the expression of innate immune response in the lungs, spleens and brains of Gal-3 KO mice compared to wild-type controls. Gene expression profiling analysis suggested that the absence of Gal-3 was associated with both enhanced and decreased expression of genes involved in the transcription of beta-glucan, mannose and chitin-responsive pattern recognition receptors (PRRs), signal transduction, inflammation, and phagocytosis. Our data suggest the importance of Gal-3 expression during innate immune responses.

Materials and methods

Animals

Six-to-eight-week-old, male C57BL/6 (wild type, WT) and Gal-3 Knockout (Gal-3 KO) mice were obtained from the animal housing facility at the Ribeirao Preto campus at the University of São Paulo (Ribeirao Preto, São Paulo, Brazil). Gal-3 KO mice were generated in the C57BL/6 mouse background as previously described and bred for nine generations [20]. The animals were housed in the animal facility at Ribeirao Preto Medical School at the University of São Paulo under optimized hygienic conditions. All animal experiments were conducted according to the Brazilian College of Animal Experimentation Protocol 100/2015 and approved by the Committee on Ethics in Animal Research at Ribeirao Preto Medical School at the University of São Paulo.

PCR array

WT and Gal-3 KO mice were euthanized and lung, spleen and brain fragments were aseptically harvested and homogenized in a tissue homogenizer (IKA® T10 basic homogenizer). Total RNA was extracted from the lung, spleen and brain homogenates using TRIzol reagent (Invitrogen Corporation, California, USA), according to the manufacturer's instructions. The concentration of the eluted total RNA was assessed by absorbance ratios (A260/A280 and A260/A230) on a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Wilmington, Delaware, USA). RNA

integrity was evaluated by electrophoresis on a 1.8% agarose gel to check the presence of the 28S and 18S bands. cDNA was synthesized by reverse transcription with oligo d(T) primers using the ImProm-IITM Reverse Transcription System Kit (Promega Corp., Fitchburg, WI). The Mouse Antifungal Response RT² Profiler PCR Array Kit (Qiagen Cat# PAMM-147Z) was used for gene expression analysis. This arrangement consisted of a plate containing 84 lyophilized primers targeting genes involved in the innate immune response to fungal infection (cited in Table S1), and negative controls for genomic DNA and primers targeting six different constituent genes. Pooled cDNA was mixed with buffer, DNA polymerase, SYBR Green Mastermix (2X RT² SYBR Green Mastermix, SABiosciences-Qiagen), and water and partitioned among the 96 wells of the plate (25 µL per well). Real-time PCR was performed in a BioRad CFX 96 thermal cycler (C1000TM thermal cycler) with the following cycling conditions: 95°C for 10 min; then, 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Ct (threshold cycle) values were analyzed using SABiosciences Web software available online through the Qiagen Data Analysis Center website.

Statistical analysis

The Ct values were normalized using selected reference genes (*gusb*, *hpert1*, *hsp90ab1*, *gapdh* and *actb*), which showed more stable expression between experimental and control groups. If more than one control gene was selected for normalization, the geometric mean of its values was performed. Our data were normalized with *b2m*, *gapdh*, *gusb*, and *hsp90ab1* for the lungs; *hsp90ab1* for the spleens, and *actb*, *b2m*, and *gusb* for the brains. The Ct values were geometrically calculated and used to determine $2^{-\Delta\Delta Ct}$. Differences in transcript levels (fold change (FC)) between experimental and control groups were determined using the Ct comparison method, based on the $2^{-\Delta\Delta Ct}$ algorithm. Genes were considered significantly modulated (induced or repressed) if the difference in the mean $2^{-\Delta\Delta Ct}$ values was greater than 2 or less than -2.

Results

Genes upregulated in Gal-3 KO mice compared to wild-type mice

The levels of expression of 84 genes present in the Mouse Antifungal Response RT² Profiler PCR Array Kit were evaluated in Gal-3 KO mice relative to WT mice. This approach helped elucidate the role of Gal-3 in modulating genes expressed during the innate immune response (Figure 1 and Table S2). A total of 19 genes were upregulated in the lungs, spleens and

Figure 1. Heat map showing expression of genes in the innate immune response in the absence of Gal-3.

Gene expression analyses in the lungs, spleens and brains of Gal-3 KO and WT mice (control) during the innate response. Ct (threshold cycle) values were transferred to SABiosciences Web software available online via the Qiagen website. Fold regulation cut off = 2 and pvalue cut off = 0,05. The scale represents fold change, with threshold set to 3 (green: upregulated, red: downregulated, black: medium change).

brains of Gal-3 KO mice, with fold regulation greater than 2. Notably, only mannose-binding lectin 2 (*mbl2*), which is involved in the initial activation of the complement system, inflammation and phagocytosis, was overexpressed in the lungs (Table S3). In the spleen, genes encoding the chemokine (C-C-motif) receptor 5 (*ccr5*), mannose receptor C type 1 (*mrc1*), and toll-like receptor 9 (*tlr9*) were upregulated (Table S4). Sixteen genes were upregulated in the brain, including complement component 3 (*c3*), caspase 1 (*casp1*), chemokines (*ccl5*, *ccl12*, *cxcl10*), *cd36*, *cd209a*, C-type lectin domain family 4 and 7 (*clec4n* and *clec7a*), colony stimulating factor 2 (*csf2*), interleukins (*il-1 β* , *il-2*, *il-12b*), mannose-binding lectin 2 (*mbl2*), pentraxin (*ptx3*), and surfactant-associated protein D (*sftpd*) (Table S5). These gene expression profiles demonstrated that absence of Gal-3 upregulates pro-inflammatory mediators and PRRs, with the notable highlight of *mbl2* upregulation in the lungs and brain (Figure 2).

Genes downregulated in Gal-3 KO mice compared to wild-type mice

The absence of Gal-3 in downregulating genes expressed in innate antifungal immunity in the lungs, spleens, and brains of Gal-3 KO mice was evaluated. Of the 84 genes tested, 42 genes were downregulated in the three types of tissues studied, with fold regulation less than -2 (Figure 1 and Table S2). In the lungs, 13 genes were downregulated in Gal-3 KO mice, including *c3*, *ccl20*, *cxcl3*, *cxcl10*, *ccr5*, *csf2*, *fcgr1*, *il-1 α* , *il-6*, *il-12a*, *il-12b*, *myd88*, and *syk*. These genes encode components of the complement system, proinflammatory mediators, chemokines and its receptors, adaptor proteins and effectors of the PRR signal transduction pathway and phagocytosis (Table S3). In the spleens of

Gal-3 KO mice, 35 genes were downregulated. These genes were categorized into PRRs (*cd36*, *cd207*, *chia1*, *clec7a*, *nptx1*, *ptx3*, *scarf1*); signal transduction (*bcl10*, *card9*, *fos*, *irak4*, *ikkbk*, *itgb2*, *jun*, *malt1*, *map2k4*, *map3k7*, *mapk14*, *syk*); inflammation (*c3*, *ccl20*, *cxcl3*, *cxcl11*, *f3*, *il-1 α* , *il-1 β* , *il-12b*, *il1r1*, *mbl2*, *myd88*); gene responsive to pathogenic fungi (*socs3*); and phagocytosis (*cd14*, *fcgr4*, *sftpd*) (Table S4). Four genes (*ccr5*, *colec12*, *fos* and *nptx1*) were downregulated in Gal-3 KO mice brains. *colec12* and *nptx1* are involved in recognition; *fos*, in the signal transduction pathways of toll-like receptors; *ccr5* and *fos*, in inflammation; and *colec12*, in phagocytosis (Table S5). Overall, most of the negatively regulated genes were found in the spleen, while *fos* and *nptx1* were also downregulated in the brain. In addition, seven genes (*c3*, *ccl20*, *cxcl3*, *il-1 α* , *il-12b*, *myd88*, *syk*), which were downregulated in Gal-3 KO mice spleens, were also downregulated in the lungs. Only the gene encoding chemokine (C-Cmotif) receptor 5 (*ccr5*) was downregulated in both lungs and brains (Figure 2).

Discussion

Gal-3 is involved in several biological processes such as angiogenesis, tumor invasion, metastasis, immune response, and maintenance of cellular homeostasis [3,13,40]. This protein is expressed in immune system cells including monocytes, macrophages, B and T lymphocytes, dendritic cells, eosinophils, and neutrophils [41,42], indicating it plays important roles in regulating the host immune response in physiological and pathological conditions [18]. In fungal infections like aspergillosis [43], candidiasis [24], cryptococcosis [22], paracoccidioidomycosis [23,25] and histoplasmosis [17] the influence of Gal-3 was analyzed through the main organs affected by these pathogens such as lungs, spleens and brains. In this study, we

Figure 2. Up- and downregulation of innate immunity genes in Gal-3 KO mice.

Comparative Venn diagram showing genes up- and downregulated in the lungs, spleens and brains of Gal-3 KO mice. *Mbl2* and *Ccr5* were up- and downregulated, respectively, in the lungs and brains. *C3*, *Ccl20*, *Cxcl3*, *Il-1 α* , *Il-12b*, *Myd88*, and *Syk* were negatively modulated in spleens and lungs. *Fos* and *Nptx1* were downregulated in spleens and brains. Venn diagram was designed using Venny 2.1.

conducted expression profiling of genes involved in the host innate immune system in lungs, spleens and brains of Gal-3 KO mice. Our results showed that the absence of Gal-3 promotes both positive and negative modulation of genes encoding PRRs that bind beta-glucan, mannose and chitin, and genes involved in signal transduction, inflammation and phagocytosis. In the lungs and spleen, these genes were preferentially negatively modulated, while in the brain genes were predominantly positively modulated.

The importance of lectins during the innate immune response has been gaining prominence [44]. The antifungal immune response involves fungal recognition by pattern recognition receptors such as C-type lectin receptors (CLRs) [45,46] will mediate the host immune response through opsonization, complement system activation, phagocytosis, and inflammation [15,47]. Also, collectins, pentraxins and complement system proteins can facilitate the engulfment of pathogens by acting as opsonins, as well as promoting direct fungicidal effects [48].

The recognition of fungal pathogens by Gal-3 can be performed in conjunction with Dectin-1, PRR responsible for the recognition of beta glucan, in which macrophages expressing these two lectins are able to produce TNF- α in response to pathogens such as *Saccharomyces cerevisiae* and *Candida albicans* [49]. Moreover, Gal-3 can promote killing of *C.albicans* by binding to beta-1,2-linked oligomannans on the cell surface [50]. Our results suggest that Gal-3 KO promotes both reduced and enhanced expression of genes involved in pathogen recognition in the absence of apathogenic stimulus. This could have implications on the immune response following a fungal infection because Gal-3, as well as other members of the galectin family, they can act as PRRs capable of discriminating the glycans present in the pathogen and host, playing an important role in immune defense [51,52].

Gal-3 may also participate in the phagocytosis of microorganisms by macrophages, triggering activation of the immune system *in vitro* [53,54]. Corroborating our results that showed the decrease in the expression of genes involved in phagocytosis, Sano et al., have verified that Gal-3 KO macrophages showed delayed phagocytosis compared to WT [55]. The addition of exogenous Gal-3 can promote the activation of macrophages in a manner dependent on the properties of this lectin. The hypothesis for the role of Gal-3 in phagocytosis would be that Gal-3 binds to the pathogen and promotes the engulfment by macrophages [55].

C-type lectins such as the mannose-binding protein (Mbl2) and galectins may play a fundamental role in neutralizing the pathogen and conducting adaptive immunity [56]. Our results demonstrated that Mbl2, which can bind to different sugars, including GlcNAc,

mannose, fucose and glucose [57], was overexpressed in lungs of Gal-3 KO compared to WT animals. The relationship between Gal-3 and Mbl2 is still unknown, but their interaction may promote several effector functions in the immune system [58].

Gal-3 deletion decreased the expression of chemokines and their receptors (*ccl20*, *cxcl1*, *cxcl3*, *cxcl10*, *cxcl11*, *ccr5*), as well as other molecules involved in inflammatory processes (*c3*, *csf2*, *il-1 α* , *il-1 β* , *il-6*, *il-12*) in the lungs and spleen. In agreement with our results, *in vitro* and *in vivo* studies suggest that Gal-3 is important for modulating inflammatory responses due to its roles in cell activation and migration or inhibition of apoptosis [6]. These observations are also supported by studies showing increased expression of galectins and chemokines in inflammatory conditions such as atherosclerosis and osteoarthritis [59–61]. Thus, our results reinforce that Gal-3 is a multifunctional protein involved in several biological events and may play crucial roles in gene expression during the host innate immune response.

Conclusion

Our results suggested that Gal-3 deletion has implications on the expression of genes involved in PRRs, signal transduction, inflammation and phagocytosis. We hypothesized that Gal-3 may also exert its effects by modulating host immunity. However, additional studies are required to better understand the mechanisms involved and identify whether Gal-3 is an attractive target for the development of therapeutic strategies to enhance antifungal immunity.

Acknowledgments

We thank Patricia Vendruscolo and Carlos Alberto Vieira for technical support.

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [420670/2018-1]; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Fundação de Apoio ao Ensino, Pesquisa e Assistência do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (FAEPA); Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [2016/03322-7, 2016/25167-3].

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References

- [1] Barondes SH, Castronovo V, Cooper DNW, et al. Galectins: a family of animal beta-galactoside-binding lectins. *Cell*. 1994 Feb;76(4):597–598. ISSN 0092-8674.
- [2] Cooper DN. Galectinomics: finding themes in complexity. *Biochim Biophys Acta*. 2002 Sep;1572(2–3):209–231. ISSN 0006-3002.
- [3] Liu FT, Rabinovich GA. Galectins as modulators of tumour progression. *Nat Rev Cancer*. 2005 Jan;5(1):29–41. ISSN 1474-175X.
- [4] Brewer CF, Miceli MC, Baum LG. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions. *Curr Opin Struct Biol*. 2002 Oct;12(5):616–623. ISSN 0959-440X.
- [5] Fred Brewer C. Binding and cross-linking properties of galectins. *Biochim Biophys Acta*. 2002 Sep;1572(2–3):255–262. ISSN 0006-3002.
- [6] Rabinovich GA, Baum LG, Tinari N, et al. Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol*. 2002 Jun;23(6):313–320. ISSN 1471-4906.
- [7] Albrandt K, Orida NK, Liu FT. An IgE-binding protein with a distinctive repetitive sequence and homology with an IgG receptor. *Proc Natl Acad Sci USA*. 1987 Oct;84(19):6859–6863. ISSN 0027-8424.
- [8] Liu FT, Patterson RJ, Wang JL. Intracellular functions of galectins. *Biochim Biophys Acta*. 2002 Sep 19;1572(2–3):263–273. ISSN 0006-3002.
- [9] Agrwal N, Wang JL, Voss PG. Carbohydrate-binding protein 35. Levels of transcription and mRNA accumulation in quiescent and proliferating cells. *JBiol Chem*. 1989;264(29):17236–17242.
- [10] Moutsatsos IK, Davis JM, Wang JL. Endogenous lectins from cultured cells: subcellular localization of carbohydrate-binding protein 35 in 3T3 fibroblasts. *JCell Biol*. 1986;102(2):477–483. ISSN 0021-9525/1540-8140.
- [11] Shimura T, Takenaka Y, Tsutsumi S, et al. Galectin-3, a novel binding partner of beta-catenin. *Cancer Res*. 2004 Sep;64(18):6363–6367. ISSN 0008-5472.
- [12] Dabelic S, Supraha S, Dumic J. Galectin-3 in macrophage-like cells exposed to immunomodulatory drugs. *Biochim Biophys Acta*. 2006 Apr;1760(4):701–709. ISSN 0006-3002.
- [13] Dumic J, Dabelic S, Flögel M. Galectin-3: an open-ended story. *Biochim Biophys Acta*. 2006 Apr;1760(4):616–635. ISSN 0006-3002.
- [14] Chung AW, Sieling PA, Schenk M, et al. Galectin-3 regulates the innate immune response of human monocytes. *J Infect Dis*. 2013;207(6):947–956. ISSN 1537-6613.
- [15] Pelka K, Nardo DE. D. emerging concepts in innate immunity. *Methods Mol Biol*. 2018;1714: 1–18. ISSN 1064-3745.
- [16] Sehrawat S, Kaur M. Galectin-3 as a modifier of anti-microbial immunity: unraveling the unknowns. *Glycobiology*. 2020;30(7):418–426. ISSN 1460-2423.
- [17] Wu SY, Yu J-S, Liu F-T, et al. Galectin-3 negatively regulates dendritic cell production of IL-23/IL-17–Axis cytokines in infection by *Histoplasma capsulatum*. *J Immunol*. 2013 Apr;190(7):3427–3437. ISSN 1550-6606. 10.4049/jimmunol.1202122
- [18] Sciacchitano S, Lavra L, Morgante A, et al. Galectin-3: one molecule for an alphabet of diseases, from A to Z. *Int J Mol Sci*. 2018 Jan 26;19(2):379. ISSN 1422-0067.
- [19] Colnot C, Sidhu SS, Balmain N, et al. Uncoupling of chondrocyte death and vascular invasion in mouse galectin 3 null mutant bones. *Dev Biol*. 2001 Jan 1;229(1):203–214. ISSN 0012-1606.
- [20] Hsu DK, Yang R-Y, Pan Z, et al. Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. *Am J Pathol*. 2000 Mar;156(3):1073–1083. ISSN 0002-9440.
- [21] Sartim AG, Sartim MA, Cummings RD, et al. Impaired emotional response to stress in mice lacking galectin-1 or galectin-3. *Physiol Behav*. 2020/06/01/2020. ISSN 0031-9384 2020;220:112862.
- [22] Almeida F, Wolf JM, Da Silva TA, et al. Galectin-3 impacts *Cryptococcus neoformans* infection through direct antifungal effects. *Nat Commun*. 2017/12/062017. ISSN 2041-1723. 2017;8(1): 1968.
- [23] Hatanaka O, Rezende CP, Moreno P, et al. Galectin-3 inhibits *paracoccidioides brasiliensis* growth and impacts *paracoccidioidomycosis* through multiple mechanisms. *mSphere*. 2019 Apr 24;4(2). ISSN 2379-5042. doi: 10.1128/mSphere.00209-19
- [24] Linden JR, De Paepe ME, Laforce-Nesbitt SS, et al. Galectin-3 plays an important role in protection against disseminated candidiasis. *Med Mycol*. 2013 Aug;51(6):641–651. ISSN 1460-2709.
- [25] Ruas LP, Bernardes ES, Fermino ML, et al. Lack of galectin-3 drives response to *Paracoccidioides brasiliensis* toward a Th2-biased immunity. *PLoS One*. 2009;4(2):e4519. ISSN 1932-6203.
- [26] Yang RY, Rabinovich GA, Liu FT. Galectins: structure, function and therapeutic potential. *Expert Rev Mol Med*. 2008 Jun;10:e17. ISSN 1462-3994.
- [27] Mendonça Belmont TFD, Do Ó KP, Soares Da Silva A, et al. Single nucleotide polymorphisms at +191 and +292 of Galectin-3 Gene (LGALS3) related to lower GAL-3 serum levels are associated with frequent respiratory tract infection and vaso-occlusive crisis in children with sickle cell anemia. *PloS One*. 2016;11(9):e0162297–e0162297. ISSN 1932-6203.
- [28] Atabaki M, Hashemi M, Daneshvar H, et al. Lectin, galactoside-binding, soluble, 3 rs4652 A/C gene variation and the risk for rheumatoid arthritis. *Biomed Rep*. 2017;6(2): 251–255. ISSN 2049-9434 2049-9442.
- [29] Tian K, Wang L, Wang K, et al. Analysis of variants at LGALS3 single nucleotide polymorphism loci in skull base chordoma. *Oncol Lett*. 2018 Jul;16(1):1312–1320. ISSN 1792-1074.
- [30] Zhang Y, Wang Y, Zhai M, et al. Influence of LGALS3 gene polymorphisms on susceptibility and prognosis of

- dilated cardiomyopathy in a Northern Han Chinese population. *Gene*. 2018 Feb 5; ISSN 0378-1119;642:293–298.
- [31] Tang T, Dai Y, Zeng Q, et al. Pentraxin-3 polymorphisms and pulmonary fungal disease in non-neutropenic patients. *Ann Transl Med*. 2020;8(18):1142. ISSN 2305-5839;2305-5847.
- [32] Zhou P, Xie Y, Yan Z, et al. Association between dectin-1 gene single nucleotide polymorphisms and fungal infection: a systematic review and meta-analysis. *Biosci Rep*. 2019;39(11):BSR20191519. ISSN 1573-4935;0144-8463.
- [33] Grube M, Loeffler J, Mezger M, et al. TLR5 stop codon polymorphism is associated with invasive aspergillosis after allogeneic stem cell transplantation. *Med Mycol*. 2013 Nov;51(8):818–825. ISSN 1369-3786.
- [34] Cunha C, Di Ianni M, Bozza S, et al. Dectin-1 Y238X polymorphism associates with susceptibility to invasive aspergillosis in hematopoietic transplantation through impairment of both recipient- and donor-dependent mechanisms of antifungal immunity. *Blood*. 2010 Dec 9;116(24):5394–5402. ISSN 0006-4971.
- [35] Sainz J, Lupiáñez CB, Segura-Catena J, et al. Dectin-1 and DC-SIGN polymorphisms associated with invasive pulmonary aspergillosis infection. *PLoS One*. 2012;7(2):e32273. ISSN 1932-6203.
- [36] Cunha C, Aversa F, Lacerda JF, et al. Genetic PTX3 deficiency and aspergillosis in stem-cell transplantation. *N Engl J Med*. 2014 Jan 30;370(5):421–432. ISSN 0028-4793.
- [37] Sainz J, Pérez E, Hassan L, et al. Variable number of tandem repeats of TNF receptor type 2 promoter as genetic biomarker of susceptibility to develop invasive pulmonary aspergillosis. *Hum Immunol*. 2007 Jan;68(1):41–50. ISSN 0198-8859.
- [38] Sainz J, Salas-Alvarado I, López-Fernández E, et al. TNFR1 mRNA expression level and TNFR1 gene polymorphisms are predictive markers for susceptibility to develop invasive pulmonary aspergillosis. *Int J Immunopathol Pharmacol*. 2010 Apr-Jun;23(2):423–436. ISSN 0394-6320.
- [39] Lupiáñez CB, Villaescusa MT, Carvalho A, et al. Common genetic polymorphisms within NFκB-related genes and the risk of developing invasive aspergillosis. *Front Microbiol*. ISSN 1664-302X 2016;7: 1243.
- [40] Nakahara S, Oka N, Raz A. On the role of galectin-3 in cancer apoptosis. *Apoptosis*. 2005/03/01 2005. ISSN 1573-675X. 2005;10(2):267–275.
- [41] Chaudhari AD, Gude RP, Kalraiya RD, et al. Endogenous galectin-3 expression levels modulate immune responses in galectin-3 transgenic mice. *Mol Immunol*. 2015 Dec;68(2 Pt A):300–311. ISSN 0161-5890.
- [42] Kouo T, Huang L, Pucsek AB, et al. Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. *Cancer Immunol Res*. 2015;3(4):412–423. ISSN 2326-6074;2326-6066.
- [43] Snarr BD, St-Pierre G, Ralph B, et al. Galectin-3 enhances neutrophil motility and extravasation into the airways during *Aspergillus fumigatus* infection. *PLoS Pathog*. 2020;16(8):e1008741.
- [44] Garred P, Genster N, Pilely K et al., A journey through the lectin pathway of complement—MBL and beyond. *Immunol Rev* 2016/11/01 2016. ISSN 0105-2896. 2016;274(1): 74–97.
- [45] Salazar F, Brown GD. Antifungal innate immunity: a perspective from the last 10 years. *J Innate Immun*. 2018;10(5–6):373–397. ISSN 1662-8128;1662-811X.
- [46] Speakman EA, Dambuza IM, Salazar F, et al. T cell antifungal immunity and the role of C-type lectin receptors. *Trends Immunol*. 2020;41(1):61–76. ISSN 1471-4981;1471-4906.
- [47] Heung LJ. Innate immune responses to *Cryptococcus*. *J Fungi (Basel)*. 2017;3(3): 35. ISSN 2309-608X.
- [48] Ma YJ, Lee BL, Garred P. An overview of the synergy and crosstalk between pentraxins and collectins/ficolins: their functional relevance in complement activation. *Exp Mol Med*. 2017;49(4):e320–e320. ISSN 2092-6413;1226-3613.
- [49] Esteban A, Popp MW, Vyas VK, et al. Fungal recognition is mediated by the association of dectin-1 and galectin-3 in macrophages. *Proc Natl Acad Sci USA*. 2011;108(34):14270–14275. ISSN 1091-6490;0027-8424.
- [50] Kohatsu L, Hsu DK, Jegalian AG, et al. Galectin-3 induces death of *Candida* species expressing specific β-1,2-linked mannans. *J Immunol*. 2006 Oct 1;177(7):4718–4726. ISSN 0022-1767.
- [51] Baum LG, Garner OB, Schaefer K, et al. Microbe–host interactions are positively and negatively regulated by Galectin–Glycan interactions. *Front Immunol*. ISSN 1664-3224 2014;5: 284.
- [52] Díaz-alvarez L, Ortega E. The many roles of Galectin-3, a multifaceted molecule, in innate immune responses against pathogens. *Mediators Inflamm*. 2017;2017:1–10.
- [53] Liu FT, Hsu DK, Zuberi RI, et al. Expression and function of galectin-3, a beta-galactoside-binding lectin, in human monocytes and macrophages. *Am J Pathol*. 1995;147(4):1016–1028. ISSN 0002-9440;1525-2191.
- [54] Sano H, Hsu DK, Yu L, et al. Human Galectin-3 is a novel chemoattractant for monocytes and macrophages. *J Immunol*. 2000;165(4):2156.
- [55] Sano H, Hsu DK, Apgar JR, et al. Critical role of galectin-3 in phagocytosis by macrophages. *J Clin Invest*. 2003;112(3):389–397. ISSN 0021-9738.
- [56] Vasta GR, Quesenberry M, Ahmed H et al., C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. *Dev Comp Immunol* 1999/06/01/1999. ISSN 0145-305X. 1999;23(4): 401–420.
- [57] Turner MW. The role of mannose-binding lectin in health and disease. *Mol Immunol*. 2003/11/01/2003. ISSN 0161-5890. 2003;40(7):423–429.
- [58] Ma YJ, Doni A, Romani L, et al. Ficolin-1-PTX3 complex formation promotes clearance of altered self-cells and modulates IL-8 production. *J Immunol*. 2013 Aug 1;191(3):1324–1333. ISSN 0022-1767.
- [59] Filer A, Bik M, Parsonage GN, et al. Galectin 3 induces a distinctive pattern of cytokine and chemokine production in rheumatoid synovial fibroblasts via selective signaling pathways. *Arthritis Rheumatism*. 2009;60(6):1604–1614. ISSN 0004-3591;1529-0131.

- [60] Nachtigal M, Ghaffar A, Mayer EP. Galectin-3 gene inactivation reduces atherosclerotic lesions and adventitial inflammation in ApoE-deficient mice. *Am J Pathol.* [2008](#);172(1):247–255. ISSN 0002-94401525-2191.
- [61] Wu S-Y, Yu J-S, Liu F-T, et al. Galectin-3 negatively regulates dendritic cell production of IL-23/IL-17–Axis cytokines in infection by *Histoplasma capsulatum*. *Journal of immunology* (Baltimore, Md.: 1950). 190 (7)03January [2013](#); doi: [10.4049/jimmunol.1202122](#).