UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO

Verônica Soares Brauer

Ação imunomoduladora de vesículas extracelulares produzidas por *Aspergillus flavus*

Ribeirão Preto
2020

VERÔNICA SOARES BRAUER

Ação imunomoduladora de vesículas extracelulares produzidas por *Aspergillus flavus*

Tese apresentada ao programa de pós-graduação em Bioquímica da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, para obtenção do título de Doutor em Bioquímica.

Área de Concentração: Bioquímica.

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

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Epígrafe

“Sou viramundo virado
Nas rondas da maravilha
Cortando a faca e facão
Os desatinos da vida
Gritando para assustar
A coragem da inimiga
Pulando pra não ser preso
Pelas cadeias da intriga
Prefiro ter toda a vida
A vida como inimiga
A ter na morte da vida
Minha sorte decidida...”

Gilberto Gil

RESUMO

BRAUER, Verônica Soares. **Ação imunomoduladora de vesículas extracelulares produzidas por *Aspergillus flavus*.** 2020. 118 f. Tese (Doutorado) - Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2020.

Dentre as centenas de espécies fúngicas pertencentes ao gênero *Aspergillus*, *A. flavus* vem se destacando devido à sua importância no âmbito da medicina e da agricultura. *A. flavus* é um fungo saprofítico, de ampla distribuição geográfica que infecta plantas e animais. Grande produtor de aflatoxinas, substâncias naturais com capacidade hepatocarcinogênica, o *A. flavus* contamina safras, principalmente de grãos, podendo causar grandes prejuízos financeiros à agroindústria, além dos problemas de intoxicação devido à ingestão dos alimentos contaminados. Em humanos, *A. flavus* é o segundo maior causador de casos de aspergilose, que devido ao seus mecanismos de virulência (enzimas hidrolíticas, metabólitos secundários, pigmentos e crescimento em altas temperaturas), consegue evadir ao sistema imune do hospedeiro, estabelecendo a infecção. Vem sendo descrito que fungos utilizam a compartimentalização dos seus fatores de virulência em estruturas chamadas “vesículas extracelulares” (EVs), com objetivo de secretá-los ao meio extracelular, modulando a interação fungo-hospedeiro. Porém, não existem estudos que demonstrem a produção de EVs por *A. flavus.* Desta forma, nosso objetivo foi avaliar a produção e caracterização de EVs por *A. flavus*, analisar se estas estruturas conseguem estimular uma resposta imunológica por macrófagos e avaliar o papel das EVs em modelo de infecção utilizando larvas de *Galleria mellonella*. Para tanto, macrófagos derivados de medula óssea (BMDMs) foram estimulados com EVs produzidas por *A. flavus,* e parâmetros como produção de mediadores inflamatórios, fagocitose, atividade microbicida e polarização foram analisados *in vitro.* Em adição, um modelo de infecção *in vivo* utilizando larvas de *G. mellonella* foi realizado a fim de analisar o papel protetor ou não das EVs. Nossos resultados demonstram que *A. flavus* produz VEs, e estas são capazes de estimular a produção de óxido nítrico (NO) e citocinas como fator de necrose tumoral (TNF)-α, interleucina (IL)-6, IL-1β. Macrófagos estimulados com EVs apresentaram maior atividade fagocítica e microbicida, acompanhada por uma polarização para o perfil M1. Por

fim, larvas tratadas com EVs previamente ao desafio com conídios de *A. flavus,* apresentaram maior sobrevivência e maior atividade microbicida. Concluímos então, que EVs produzidas por *A. flavus* são estruturas bioativas, com capacidade imunogênica, podendo modular a interação entre o fungo e o hospedeiro, auxiliando no controle e eliminação da infecção, evitando o estabelecimento de quadros de aspergilose.

Palavras-chave: *Aspergillus flavus*. Vesículas extracelulares. Macrófagos. Citocinas. Fagocitose. Atividade microbicida. *Galleria mellonella*.

ABSTRACT

BRAUER, Verônica Soares. **Immunomodulatory actions of extracellular vesicles produced by *Aspergillus flavus*.** 2020. 118 f. Tese (Doutorado) - Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo, Ribeirão Preto, 2020.

Among Among the several hundred fungal species belonging to the *Aspergillus* genus, the *A. flavus* have been recognized by its importance on medical and agriculture ambit. The *A. flavus* is a saprophytic fungus, with wide geographical distribution that infects plants and animals. Major aflatoxin producer, natural substances with hepatocarcinogenic action, *A. flavus* can contaminate crops, mainly of grains, causing huge economical losses to agroindustry, in addition to intoxication problems caused by ingested contaminated food. In humans, *A. flavus* is the second most common cause of aspergillosis, since it's several virulence factors (as hydrolytic enzymes, secondary metabolites, pigments, growth at high temperatures) allows the fungus to evade the host immune system, establishing the infection. It has been described that the fungi use the compartmentalization of its virulence factors on structures called by "extracellular vesicles" (EVs), in order to secret them on extracellular space, modulating the fungus-host interaction. But, there is no studies demonstrating the EV production by *A. flavus*. So, our aim was evaluate the EV production by *A. flavus,* evaluate if these structures are able to induce an immune response on macrophages and the influence of EVs during *A. flavus* infection in *Galleria mellonella* larvae. For that, bone-marrow derived macrophages (BMDMs) were stimulated with *A. flavus* EVs, and parameters of inflammatory mediators production, phagocytosis, killing, and polarization were analyzed *in vitro*, and we analyzed the possible EV protector role using *G. mellonella in vivo* model. Our results demonstrated that *A. flavus* were able to produce EVs, and these structures were able to stimulate nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β production. Macrophages stimulated with EVs showed higher phagocytic and microbicidal activity followed by M1 polarization than untreated macrophages. Finally, *G. mellonella* larvae treated with EVs previously *A. flavus* conidia challenge, demonstrated higher survival rates and higher killing activity than untreated larvae. We concluded that EVs produced by *A. flavus* are a bioactive structure with immunogenic ability, able to modulates the fungus-host interaction and

helping in the control and elimination of fungal infection, avoiding the establishment of aspergillosis.

Keywords: *Aspergillus flavus*. Extracellular vesicles. Macrophage. Cytokine. Phagocytosis. Killing. Polarization. *Galleria mellonella.*

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LISTA DE ABREVIATURAS

$(NH_4)_2MoO_4$: *Ammonium molybdate*

µl: Microlitro

µm: Micrômetro

***A. flavus*:** *Aspergillus flavus*

A. fumigatus: *Aspergillus fumigatus*

ANVISA: Agência Nacional de Vigilância Sanitária

Arg-1: Arginase 1

BMDMs: *Bone marrow-derived macrophages*

C. albicans: *Candida albicans*

C. neoformans: *Cryptococcus neoformans*

CCL2: *Chemokine (C-C motif) ligand 2*

cDNA: *Complementary DNA*

CFU: *Colony forming unit*

$CoCl_2$: *Cobalt dichloride*

$CuSO_4$: *Cupric sulfate*

DC-SIGN: Ligante de molécula de adesão intercelular não integrina específica de células dendríticas

DMEM: *Dulbecco's modified Eagle medium*

DNA: *Deoxyribonucleic acid*

EDTA: *Ethylenediaminetetraacetic acid*

ELISA: *Enzyme linked immunosorbent assay*

ERN: Espécies reativas de nitrogênio

EROs: Espécies reativas de oxigênio

EVs: *Extracellular vesicles*

FBS: *Fetal bovine serum*

FDA: *Food and Drug Administration*

$FeSO_4$: *Iron (II) sulfate*

FIZZ-1: *Inflammatory zone 1 /* encontrada na zona inflamatória 1

***G. mellonella*:** *Galleria mellonella*

GXM: Glucoronoxilomanana

H. capsulatum: *Histoplasma capsulatum*

H_2O: *Water*

H_3BO_3: *Boric acid*

H_3PO_4: *Phosphoric acid*

HIV: *Human immunodeficiency virus*

IFN-γ: *Gamma interferon*

IL-: Interleucina/*Interleukin*

iNOS: *Inducible nitric oxide synthase*

KCL: *Potassium chloride*

kDa: Kilodalton

KH_2PO_4: *Potassium phosphate monobasic*

LPS: *lipopolysaccharide*

MCP-1: Proteína quimioatraente de monócito 1

mg: miligramas

$MgSO_4$: *Magnesium sulfate*

MHC: Complexo principal de histocompatibilidade

MIP-1α: proteína inflamatória de macrófago 1 alfa

ml: Mililitro

mm: Milímetro

$MnCl_2$: *Manganese dichloride*

mRNA: *Messenger RNA*

$NaNO_2$: *Sodium nitrite*

$NaNO_3$: *Sodium nitrate*

NaOH: *Sodium hydroxide*

NADPH: Nicotinamida Adenina Dinucleotídeo Fosfato

ng: Nanogramas

nm: *nanometers*

NO: *Nitric Oxide*

NTA: *Nanoparticle tracking analysis*

P. brasiliensis: *Paracoccidioides brasiliensis*

PBS: *Phosphate-buffered saline*

PCR: *Polymerase chain reaction*

PDA: *Potato dextrose Agar*

PKS: Policétido sintase

qRT-PCR: *Quantitative reverse transcription-PCR*

RNA: *Ribonucleic acid*

rpm: *Rotations per minute*

RPMS-USP: *Ribeirao Preto Medical School at the University of Sao Paulo*

S. brasiliensis: *Sporothrix brasiliensis*

SD: *Standard deviation*

SOCS3: Supressor de sinalização de citocina 3

TGF-β: Fator de crescimento transformante β

TLR: *Toll like rececptors*

TNF-α: *Tumor necrosis factor alpha*

T. interdigitale: *Trichophyton interdigitale*

WT: *Wild-type*

$ZnSO_4$: *Zinc sulfate*

SUMÁRIO

Capítulo I - Considerações Gerais

1. Introdução

1.1 O GÊNERO *Aspergillus*

Fungos pertencentes ao gênero *Aspergillus* (família *Aspergillaceae,* ordem *Eurotiales,* classe Eurotiomycetes, filo *Ascomycota*), são reconhecidos por sua relevância social e econômica (HOUBRAKEN; DE VRIES; SAMSON, 2014; SAMSON; VISAGIE; HOUBRAKEN; HONG *et al.*, 2014). Este gênero é composto por aproximadamente 250 espécies de fungos filamentosos, saprofíticos, presentes em solo, água, ar, e matéria orgânica em decomposição (DE VALK; KLAASSEN; MEIS, 2008). Foram assim nomeados em 1729, pelo padre biólogo Pier Antonio Micheli, que ao analisar características microscópicas dos conidióforos, as relacionou com os aspersórios (do latim *aspergere*), objetos utilizados por sacerdotes no catolicismo para aspergir água benta (BENNETT, 2010).

A identificação e classificação dos fungos do gênero *Aspergillus* ocorrem com base em características morfológicas, moleculares e quimiotaxonomia (SAMSON; VISAGIE; HOUBRAKEN; HONG *et al.*, 2014). As espécies são caracterizadas por possuírem uma estrutura contendo esporos assexuados (ou conídios) chamada de “conidióforo”, representada na Figura 1. O conidióforo é caracterizado por possuir em sua base, uma estrutura em forma de “T” ou “L”, conhecida como “célula-pé”, de onde se origina uma haste alongada que culmina em região bulbosa chamada de “vesícula” (BENNETT, 2009; BENNETT, 2010). A partir da vesícula uma ou duas camadas de células se originam, as chamadas esterigma primária e secundária, respectivamente (ou também, métula e fiálide, respectivamente), onde milhares de esporos por conidióforo são produzidos a partir das fiálides, dispostos em cadeias não ramificadas, organizadas de maneira colunar ou radial, unidos por pontes conectivas que podem ser conspícuas ou invisíveis (GUGNANI, 2003; KRIJGSHELD; BLEICHRODT; VAN VELUW; WANG *et al.*, 2013). É importante ressaltar que a presença de célula-pé, a forma e tamanho das vesículas, assim como a disposição, cor e tamanho dos esporos são importantes características utilizadas para a distinção das espécies pertencentes ao gênero *Aspergillus* (BENNETT, 2009; BENNETT, 2010; GUGNANI, 2003; KRIJGSHELD; BLEICHRODT; VAN VELUW; WANG *et al.*, 2013; PARK; JUN; HAN; HONG *et al.*, 2017; SAMSON, 1994). Em adição, aspectos da colônia (cor, diâmetro, características do micélio vegetativo, pigmentação do reverso e ao redor da colônia),

presença de estruturas de reprodução sexuada (cleistotécio, células Hulle e esclerótia), caracterização molecular (identificação de marcadores como, por exemplo, *Internal Transcriber Spacer* (ITS) e calmodulina) e produção de metabólitos secundários também são empregados na identificação e classificação das espécies (BENNETT, 2010; SAMSON, 1994; SAMSON; VISAGIE; HOUBRAKEN; HONG *et al.*, 2014), que são divididas em 4 subgêneros e 19 seções, de acordo Houbraken *et al,* 2014, representadas na Tabela 1 (HOUBRAKEN; DE VRIES; SAMSON, 2014).

Figura 1 – Representação do conidióforo, estrutura característica de fungos pertencentes ao gênero *Aspergillus*.

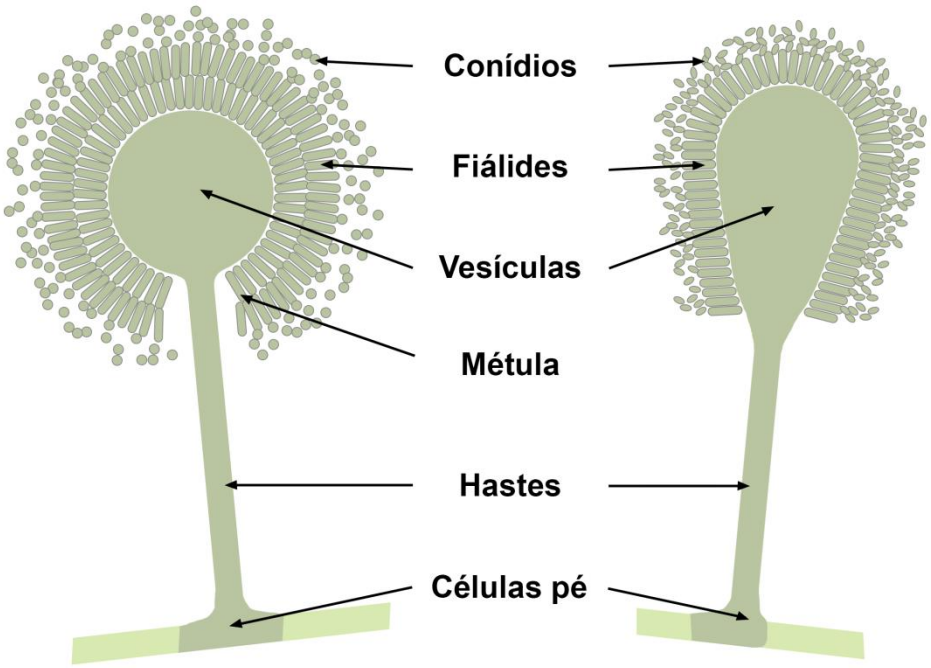


Tabela 1 – Classificação das espécies fúngicas do gênero *Aspergillus* (GAUTIER; NORMAND; RANQUE, 2016; HOUBRAKEN; DE VRIES; SAMSON, 2014; PARK; JUN; HAN; HONG *et al.*, 2017; SAMSON; VISAGIE; HOUBRAKEN; HONG *et al.*, 2014).

Subgênero	Seção	Exemplo de Espécies
Aspergillus	*Aspergillus*	*A. glaucus*
	Restricti	*A. restrictus*
Circumdati	*Candidi*	*A. candidus*
	Circumdati	*A. ochraceus*
	Flavi	*A. flavus*
	Flavipedes	*A. flavipes*
	Nigri	*A. niger*
	Terrei	*A. terreus*
Fumigati	*Cervini*	*A. cervinus*
	Clavati	*A. clavatus*
	Fumigati	*A. fumigatus*
Nidulantes	*Aenei*	*A. aeneus*
	Bispori	*A. bisporus*
	Cremei	*A. wentii*
	Nidulantes	*A. nidulans*
	Ochraceorosei	*A. ochraceoroseus*
	Silvati	*A. silvaticus*
	Sparsi	*A. panamensis*
	Usti	*A. ustus*

As espécies do gênero *Aspergillus*, como resultado do seu metabolismo primário e secundário, produzem diversos compostos de relevância econômica para agricultura, indústrias alimentares, de bebidas, enzimas e farmacêuticas, assim como, para saúde humana (BENNETT, 2010; GUGNANI, 2003). Exemplos clássicos são a utilização do *A. niger* para produção de ácido cítrico (BENNETT, 2009; PARK; JUN; HAN; HONG *et al.*, 2017); Na Ásia, *A. oryzae* e *A. sojae* são utilizados para

fermentação alimentar e produção de saquê, missô, shoyo (BENNETT, 2009); E uso farmacológico de estatinas (substância utilizada para controle sérico do colesterol), visto que a primeira estatina desenvolvida para uso humano foi produzida a partir do metabolismo secundário do *A. terreus* (BAKER; BENNETT, 2008).

A. flavus e *A. parasiticus* podem ser reconhecidos pela capacidade de produção de um grupo de micotoxinas chamadas “aflatoxinas”, substâncias tóxicas resultantes do metabolismo secundário, com ação hepatotóxica, teratogênica e relevante toxicidade ao sistema imunológico (KUMAR; MAHATO; KAMLE; MOHANTA *et al.*, 2017). Por fim, *A. fumigatus* é amplamente reconhecido por sua capacidade de causar infecções em humanos, resultando em quadros clínicos de aspergilose (SUGUI; KWON-CHUNG; JUVVADI; LATGÉ *et al.*, 2014).

1.2 *Aspergillus flavus*

1.2.1 Aspectos Gerais

Pertencente ao subgênero *Circumdati* e seção *Flavi*, *A. flavus* foi descrito pela primeira vez em 1806, e desde então, vem sendo reconhecido por ser uma das espécies mais isoladas no âmbito da agricultura e medicina, classificado com um dos dez fungos mais temidos em todo planeta (AMAIKE; KELLER, 2011; CLEVELAND; YU; FEDOROVA; BHATNAGAR *et al.*, 2009; HYDE; AL-HATMI; ANDERSEN; BOEKHOUT *et al.*, 2018).

Morfologicamente, quando cultivado em Agar Extrato de Levedura Czapek, *A. flavus* é caracterizado por possuir rápido crescimento em ambas as temperaturas de 25°C e 37°C, produz conídios de cor verde-amarelados, e colônias com 65-70 mm de diâmetro após sete dias de cultura a 25°C (JACKSON; DOBSON, 2016; KLICH, 2007). O aspecto da colônia é plano, flocoso a granular, com hifas hialinas, septadas e ramificadas a 45º, e conídios sendo produzidos a partir do micélio basal (GUGNANI, 2003; JACKSON; DOBSON, 2016; KRISHNAN; MANAVATHU; CHANDRASEKAR, 2009). Alguns isolados são capazes de produzir esclerócio, com aspecto globoso e coloração branca que gradualmente vai modificando para

marrom-avermelhado até a cor preta, com formato esférico possuindo 400 - 800 µm de diâmetro (JACKSON; DOBSON, 2016). As cabeças conidiais são tipicamente radiais, com variações de tamanho e forma, haste possuindo 400-800 µm de tamanho, vesículas de forma esféricas a subesferoidais com diâmetro entre 20-45 µm (mas podendo alcançar 50 µm) e possuindo ambas métulas e fiálides (porém, alguns isolados podem apresentar somente fiálides) onde conídios com diâmetro de 3-6 µm são produzidos e organizados em cadeias radiais (GUGNANI, 2003; HEDAYATI; PASQUALOTTO; WARN; BOWYER *et al.*, 2007; JACKSON; DOBSON, 2016; KLICH, 2007; KRISHNAN; MANAVATHU; CHANDRASEKAR, 2009).

Devido à alta produção e dispersão dos conídios, o *A. flavus* é amplamente distribuído geograficamente (HEDAYATI; PASQUALOTTO; WARN; BOWYER *et al.*, 2007), sendo isolado principalmente a partir do ar, solo, forragem e vegetação em decomposição de zonas climáticas quentes (latitudes entre 16° e 35°) (AMAIKE; KELLER, 2011; GOURAMA; BULLERMAN, 1995; PAYNE; YU; NIERMAN; MACHIDA *et al.*, 2008). No solo, estão presentes na estrutura de conídios ou esclerócio, e em plantas, como micélio (AMAIKE; KELLER, 2011), sendo considerado um patógeno de plantas e animais (PAYNE; YU; NIERMAN; MACHIDA *et al.*, 2008).

Em relação às culturas agrícolas, a infecção por *A. flavus* pode ocorrer durante a produção, colheita, estocagem e processamento dos alimentos (WILLIAMS; PHILLIPS; JOLLY; STILES *et al.*, 2004). Este fungo infecta safras especialmente de grãos ricos em óleos, como milho, amendoim, nozes e semente de algodão, onde em detrimento de seu metabolismo secundário, produzem aflatoxinas (AMAIKE; KELLER, 2011; PAYNE; YU; NIERMAN; MACHIDA *et al.*, 2008). Quimicamente, as aflatoxinas são derivadas da difuranocumarina que de acordo com a cor da fluorescência sob luz ultravioleta (azul ou verde) e perfil de migração em cromatografia de camada delgada, são classificadas em quatro grupos principais: B_1, B_2, G_1 e G_2 (KLICH, 2007; YU; PAYNE; CAMPBELL; GUO *et al.*, 2008). Por ordem de toxicidade a aflatoxina B_1 é a mais tóxica, seguida de G_1, B_2 e G_2 (KUMAR; MAHATO; KAMLE; MOHANTA *et al.*, 2017), sendo o *A. flavus* produtor apenas da B_1 e B_2 (PAYNE; YU; NIERMAN; MACHIDA *et al.*, 2008). Como resultado da ingestão de alimento ou ração contaminada, quadros de aflatoxicose ocorrem,

sendo a aflatoxicose aguda resultante da ingestão de altas concentrações de aflatoxinas em poucas exposições, levando a sintomas clínicos como icterícia, edema nos membros, dor, vômito, necrose, cirrose, falha hepática e morte (AMAIKE; KELLER, 2011; YU; PAYNE; CAMPBELL; GUO *et al.*, 2008). Por outro lado, a aflatoxicose crônica resulta da ingestão diária de menores concentrações de aflatoxina, causando perda de apetite, crescimento atrofiado, carcinoma hepatocelular e imunossupressão (AMAIKE; KELLER, 2011; KLICH, 2007; WILLIAMS; PHILLIPS; JOLLY; STILES *et al.*, 2004). Devido a esses fatores que agências reguladoras, como por exemplo, *Food and Drug Administration* (FDA) dos EUA e Agência Nacional de Vigilância Sanitária (ANVISA) do Brasil dispõem de regulamentações para limites de micotoxinas tolerados em alimentos (ANVISA, 2011; FOOD AND DRUG ADMINISTRATION, 2019; WILLIAMS; PHILLIPS; JOLLY; STILES *et al.*, 2004).

Em humanos e animais, a infecção por espécies de *Aspergillus* é chamada de “aspergilose” (AMAIKE; KELLER, 2011). Com base em síndromes clínicas, a aspergilose pode ser classificada como alérgica (quadros de asma alérgica, alveolite alérgica extrínseca, aspergilose broncopulmonar alérgica), saprofítica (não causa uma resposta imune relevante, é vista em situações de aspergiloma, aglomerados de característica esférica composta de micélio fúngico) ou invasiva (geralmente ocorre em pacientes imunodeprimidos, sendo pulmões, sinos paranasais e sistema nervoso central os sítios mais comuns de infecção) (AMAIKE; KELLER, 2011; RUDRAMURTHY; PAUL; CHAKRABARTI; MOUTON *et al.*, 2019; WARRIS, 2014). Dentre as centenas de espécies fúngicas pertencentes ao gênero *Aspergillus,* aproximadamente 20 são capazes de causar doenças em humanos, sendo o *A. flavus* a segunda maior causadora de aspergilose invasiva e não-invasiva, precedida apenas por *A. fumigatus* (PASQUALOTTO, 2009). Devido à capacidade de sobrevivência do *A. flavus* em regiões quentes e áridas, a predominância das infecções ocorrem na Ásia, Oriente Médio e África, correspondendo cerca de 50-80% dos casos de sinusite, queratite e infecções cutâneas (RUDRAMURTHY; PAUL; CHAKRABARTI; MOUTON *et al.*, 2019). No Brasil, análises realizadas em 133 isolados clínicos previamente identificados como *Aspergillus spp.,* coletados em 12 centros médicos dos país entre 2006 e 2013, revelaram que 22% dos isolados

foram identificados como *A. flavus,* representando a segunda espécie mais prevalente do estudo, antecedida apenas por *A. fumigatus* (com 53% dos casos) (NEGRI; GONÇALVES; XAFRANSKI; BERGAMASCO et al., 2014).

Um dos principais mecanismos utilizados para infecção por *A. flavus* é a produção e dispersão de conídios. Devido à característica de ser um fungo saprofítico, conídios produzidos por *A. flavus* são amplamente encontrados em solo, água, grãos, matéria orgânica em decomposição, e também, no ar (HEDAYATI; PASQUALOTTO; WARN; BOWYER *et al.*, 2007). A quantidade de conídios detectados em ambientes internos e externos de hospitais, e até mesmo, em lares, são considerados importantes fatores determinantes para desenvolvimento de surtos de aspergilose (RUDRAMURTHY; PAUL; CHAKRABARTI; MOUTON et al., 2019), visto que rotineiramente humanos inalam centenas de conídios (LASS-FLÖRL; ROILIDES; LÖFFLER; WILFLINGSEDER *et al.*, 2013), sendo esta, a principal via de acesso para infecção do hospedeiro. Em adição à inalação, secundariamente os conídios do *A. flavus* adentram ao hospedeiro através de feridas, soluções endovenosas contaminadas, curativos, fumo de plantas contaminadas (como tabaco e maconha), e infecções nosocomiais (AMAIKE; KELLER, 2011; KRISHNAN; MANAVATHU; CHANDRASEKAR, 2009). É importante destacar que via ação de defesa do epitélio mucociliar e de fagócitos residentes, geralmente os conídios são eliminados sem acarretar quaisquer danos ao hospedeiro (LASS-FLÖRL; ROILIDES; LÖFFLER; WILFLINGSEDER *et al.*, 2013; VANDENBERGH; VERWEIJ; VOSS, 1999), porém, condições como neutropenia, tratamento com agentes imunossupressores, altas doses de corticosteroides ou leucemias, o desenvolvimento da infecção é favorecido, resultando em quadros de aspergilose invasiva (KRAPPMANN, 2008; LASS-FLÖRL; ROILIDES; LÖFFLER; WILFLINGSEDER *et al.*, 2013; VANDENBERGH; VERWEIJ; VOSS, 1999; WARRIS, 2014).

Uma vez que os conídios adentraram ao hospedeiro, diversas características bioquímicas, celulares e morfológicas do fungo ditam o estabelecimento da infecção, como: termotolerância, características morfológicas conidiais, capacidade de adaptação metabólica ao ambiente, produção de proteinases, pigmentos, proteínas,

metabólitos secundários e evasão ao sistema imune do hospedeiro (AMAIKE; KELLER, 2011; DENHAM; WAMBAUGH; BROWN, 2019).

Um dos fatores mais importantes que rege a patogenicidade do fungo é a capacidade de germinar e sobreviver em temperaturas ≥37ºC (KOHLER; CASADEVALL; PERFECT, 2014). O *A. flavus* é capaz de germinar a 37ºC, e tem seu crescimento relatado em temperaturas que vão de 12°C a 48°C, porém, 37ºC é considerada a temperatura ideal de crescimento, favorecendo assim, o desenvolvimento da infecção em humanos (ARAUJO; RODRIGUES, 2004; HEDAYATI; PASQUALOTTO; WARN; BOWYER *et al.*, 2007).

Em adição à capacidade de germinação do conídio à 37ºC, sua morfologia dita o desenvolvimento dos diferentes quadros de aspergilose no hospedeiro (KRAPPMANN, 2008). Comparando com *A. fumigatus*, conídios produzidos por *A. flavus* possuem maior tamanho (2-3,5 µm comparados com 3-6 µm, respectivamente) (PASQUALOTTO, 2009), favorecendo o depósito destes no trato respiratório superior do hospedeiro, resultando em quadros de sinusite fúngica (HEDAYATI; PASQUALOTTO; WARN; BOWYER *et al.*, 2007; PASQUALOTTO, 2009). Contrariamente, devido ao menor tamanho dos conídios do *A. fumigatus*, estes alcançam com maior facilidade os alvéolos pulmonares, onde podem germinar resultando na formação de hifas, responsáveis pela difusão fúngica por outros tecidos, além do pulmonar, e assim, aspergilose invasiva (HEDAYATI; PASQUALOTTO; WARN; BOWYER *et al.*, 2007; KRAPPMANN, 2008; PASQUALOTTO, 2009).

De acordo com o curso da infecção, o fungo está sujeito a diversas variações ambientais, dentro do hospedeiro, sendo importantíssima a capacidade de se adaptar e sobreviver em diferentes microambientes, para que a infecção se estabeleça (ABAD; FERNANDEZ-MOLINA; BIKANDI; RAMIREZ *et al.*, 2010). Para isso, ações como obtenção de nutrientes a partir da degradação de macromoléculas e, degradações de barreiras estruturais são realizadas por meio de enzimas extracelulares secretadas pelo fungo. O *A. flavus* é dotado de um vasto arsenal de enzimas degradativas como α-amilase, pectinase, proteinase, amilase, cutinase, lípase e elastase, que auxiliam nos processos supracitados e, também na virulência

da cepa (AMAIKE; KELLER, 2011; HOGAN; KLEIN; LEVITZ, 1996; KRISHNAN; MANAVATHU; CHANDRASEKAR, 2009; MELLON; COTTY; DOWD, 2007; PASQUALOTTO, 2009). Por exemplo, cepas de *A. flavus* produtoras de elastase estão diretamente correlacionadas com o desenvolvimento de aspergilose invasiva em humanos, visto que o pulmão é composto por aproximadamente 30% de elastina e o fungo secreta esta enzima com objetivo de degradar este componente estrutural, facilitando o processo de invasão tecidual (HOGAN; KLEIN; LEVITZ, 1996; KOLATTUKUDY; LEE; ROGERS; ZIMMERMAN *et al.*, 1993; MELLON; COTTY; DOWD, 2007). Da mesma forma, enzimas que degradam componentes da parede celular vegetal, são empregadas por *A. flavus* para o processo de infecção, como por exemplo as pectinases (MELLON; COTTY; DOWD, 2007). *A. flavus* possui várias enzimas com atividade pecnolítica, como P1, P2c, P3 e pectinametiltransferase. Através da eliminação da expressão do gene que codifica a P2c (*pecA*), em modelo de infecção utilizando "*cotton bolls*", é demonstrado que esta enzima está associada ao aumento da invasão e colonização fúngica, refletindo assim, na virulência de *A. flavus* (SHIEH; BROWN; WHITEHEAD; CARY *et al.*, 1997).

Outra importante característica do *A. flavus* é a produção de pigmentos, compostos presentes em hifas, ascóporos, conídios e esclerócios, sendo estes dois últimos geralmente dotados do pigmento melanina (CHANG; CARY; LEBAR, 2020). A melanina proporciona ao fungo habilidade de lidar com condições ambientais adversas, como calor, exposição à radiação ionizante, luz ultravioleta, dissecação, estresse oxidativo e resistência à fagocitose pelos macrófagos, células da imunidade inata do hospedeiro (BELOZERSKAYA; GESSLER; AVER'YANOV, 2017; PASQUALOTTO, 2009), fornecendo ao fungo vantagens internamente ou externamente ao hospedeiro, caracterizando-se um fator de virulência (KRISHNAN; MANAVATHU; CHANDRASEKAR, 2009). Uma ilustração da participação de pigmentos para a proteção e resistência fúngica, se dá a partir da deleção do gene codificador da policétido sintase (PKS) do cluster 27 (*pks27*) do *A. flavus*, resultando na produção de esclerócios de coloração cinza-amarelada, em contraste com a coloração preta dos esclerócios produzidos pelo *A. flavus* selvagem. Como consequência, os esclerócios produzidos pela cepa mutante, além da mudança de

pigmentação se tornaram mais suscetíveis à predação por insetos, ao calor e luz ultravioleta, quando comparados aos esclerócios do *A. flavus* selvagem, demonstrando que alteração na pigmentação resultou em maior suscetibilidade dos esclerócios a condições ambientais extremas, reforçando a importância dos pigmentos para a fisiologia e sobrevivência fúngica (CARY; HARRIS-COWARD; EHRLICH; DI MAVUNGU *et al.*, 2014; CHANG; CARY; LEBAR, 2020).

Em adição, Cary e colaboradores, 2014, com a deleção da *pks27* demonstraram que o pigmento presente nos esclerócios do *A. flavus* não é derivado do processo de melanização, e sim, são antraquinonas (mais especificamente *asparasone A*) produzidas por enzimas Pks27 (CARY; HARRIS-COWARD; EHRLICH; DI MAVUNGU *et al.*, 2014). As enzimas PKS são importantes participantes no metabolismo secundário do *A. flavus*, sendo relacionadas à produção de compostos como aflatoxinas (ações hepatotóxicas), *aflavarin* (composto relacionado com a produção de esclerócios e ação anti-insetos), e o supracitado, *asparasone A* (funções de pigmentação do esclerócio, resistência a condições de estresse e ação anti-insetos) (CARY; GILBERT; LEBAR; MAJUMDAR *et al.*, 2018). Apesar dos metabólitos secundários serem definidos como “não essenciais” ao fungo, eles são extremamente bioativos, podendo interferir com os mecanismos de sobrevivência fúngica, seja interna ou externamente ao hospedeiro (CARY; GILBERT; LEBAR; MAJUMDAR *et al.*, 2018). Em adição ao *asparasone A*, o papel da aflatoxina no processo de patogenicidade e virulência do *A. flavus* ainda é motivo de discussão, primeiramente porque apesar de já ter sido demonstrado que *A. flavus* é capaz de produzir aflatoxinas durante quadro de aspergilose (MORI; MATSUMURA; YAMADA; IRIE *et al.*, 1998), cepas não-aflatoxigências também são capazes de causar infecção (LEEMA; KALIAMURTHY; GERALDINE; THOMAS, 2010). Em adição, estudos sugerem uma ação secundária das aflatoxinas nesse processo de virulência e patogênicidade de *A. flavus*, visto que estes compostos inibem funções efetoras de neutrófilos (SILVOTTI; PETTERINO; BONOMI; CABASSI, 1997), monócitos (CUSUMANO; ROSSANO; MERENDINO; ARENA *et al.*, 1996), macrófagos (CUSUMANO; COSTA; SEMINARA, 1990; JAKAB; HMIELESKI; ZARBA; HEMENWAY *et al.*, 1994), natural *killer* (NK), inibe populações de células linfocitárias (MEISSONNIER; PINTON; LAFFITTE; COSSALTER *et al.*,

2008) e inibe o transporte mucociliar do hospedeiro, um dos primeiros mecanismos de defesa do trato respiratório contra infecções (LEE; WORKMAN; CAREY; CHEN *et al.*, 2016).

Em modelos experimentais *in vivo* de aspergilose, *A. flavus* se mostra extremamente patogênico. Ford e Friedman, 1967, comparando a patogênicidade de 14 espécies de *Aspergillus* inoculando via endovenosa diferentes concentrações de conídios (10^2, 10^4, 10^6/ml) em camundongos não-imunocomprometidos, demonstraram que *A. flavus* é capaz de matar 100% dos animais dentro de cinco dias após inoculação de 10^6 conídios, e 38% dos animais dentro de 5 a 10 dias após inoculação de 10^4 conídios, demonstrando ser, dentre as 14 testadas, uma das espécies com maior virulência (apenas o *A. tamarii* apresentou virulência marginalmente superior ao *A. flavus*) (FORD; FRIEDMAN, 1967). Corroborando com estes dados, Mosquera *et al*, 2001, demonstram que a dose letal (DL90) do *A. flavus* é cerca de 4-50 vezes menor do que a DL90 de *A. fumigatus,* e 40-100 vezes menor do que o *A. terreus* (MOSQUERA; WARN; MORRISSEY; MOORE *et al.*, 2001), reafirmando a alta virulência de *A. flavus* em modelos animais de aspergilose. É importante destacar que apesar da alta virulência, o perfil de resposta imune frente à infecção por *A. flavus* não é completamente elucidado. Anand e colaboradores, 2010, demonstraram a infecção de camundongos via intratraqueal com conídios de *A. flavus* se dá em duas fases: a primeira caracterizada pela alta taxa de eliminação fúngica nos pulmões através da ação de macrófagos alveolares residentes e neutrófilos recrutados da corrente sanguínea, com concomitante produção de citocinas pró-inflamatórias. E a segunda fase é caracterizada por ação de monócitos recrutados da corrente sanguínea que agem eliminando debris celulares e fungos remanescentes, acompanhado de redução dos níveis de citocinas pró-inflamatórias. Os dados de secreção de citocinas mostraram que citocinas inflamatórias como IFN-γ mostram uma maior concentração 24h pós-infecção, e depois, começam declinar. Os níveis de TNF-α se mostraram inversamente proporcionais aos níveis de IL-10, e por fim os níveis de IL-4 foram maiores após 6 h da infecção, e tenderam a diminuição 12-48h pós-infecção. Esses dados demonstram que durante a infecção, a produção de citocinas é dinâmica, sendo ambas de padrão de resposta imune Th1 e Th2 produzidas (ANAND; TIWARY, 2010).

1.2.2 Macrófagos e seus mecanismos efetores contra *A. flavus*

O sistema imunológico é dotado de diversos mecanismos que abrangem respostas imunes inatas e adaptativas, que auxiliam na eliminação das centenas de conídios de *A. flavus* inalados diariamente. Dentre estes mecanismos, citam-se defesas do epitélio mucociliar, processos fagocíticos, produção de mediadores inflamatórios, ativação de células T, dentre outros (LASS-FLÖRL; ROILIDES; LÖFFLER; WILFLINGSEDER *et al.*, 2013).

A primeira barreira que o conídio encontra ao adentrar o sistema respiratório do hospedeiro é o sistema mucociliar. As vias aéreas são constituídas por uma mucosa composta de células epiteliais ciliadas, que em adição à produção de muco, fornecem um importante mecanismo de defesa contra patógenos invasores, alérgenos, debris e toxinas (BUSTAMANTE-MARIN; OSTROWSKI, 2017; SEARS; YIN; OSTROWSKI, 2015). Porém, conídios produzidos por *A. flavus* podem ultrapassar essa barreira de células ciliadas e alcançar os pulmões estimulando o desenvolvimento de respostas de células da imunidade inata do hospedeiro (PARK; MEHRAD, 2009).

Os macrófagos alveolares, por serem os fagócitos mais abundantes no pulmão, constituem a principal linha de defesa do hospedeiro contra os conídios inalados de *A. flavus* (SEGAL, 2007). É demonstrado que este tipo de macrófago é mais efetivo na prevenção de germinação de conídio de *A.* flavus quando comparados, por exemplo, a macrófagos peritoneais (SCHAFFNER; DOUGLAS; BRAUDE; DAVIS, 1983). Os macrófagos são células mononucleares do sistema imune, que estão distribuídos estrategicamente pelos tecidos corporais a fim de ingerir e degradar patógenos, células mortas e debris, orquestrando o desenvolvimento de um processo inflamatório (ABBAS; LITCHMAN; PILLAI, 2011; VAROL; MILDNER; JUNG, 2015). Para que os macrófagos consigam exercer tais funções, é necessário o reconhecimento de Padrões Moleculares Associados aos Patógenos (PAMPs), pelos diversos Receptores de Reconhecimento Padrão (PRRs) que este fagócito mononuclear possui (ABBAS; LITCHMAN; PILLAI, 2011). *Toll-like receptor* (TLR)2, TLR4, receptores de lectina tipo C (Dectina-1), receptores de manose, ligante de molécula de adesão intercelular não integrina específica de

células dendríticas (DC-SIGN) são os principais PRRs relacionados com resposta à fungos do gênero *Aspergillus*, que reconhecem constituintes como: β-glucana, manana, quitina, galactomanana (LASS-FLÖRL; ROILIDES; LÖFFLER; WILFLINGSEDER *et al.*, 2013; SEGAL, 2007). Esse dado é confirmado pelo fato de que pulmões de camundongos infectados por *A. flavus* expressam mais TLR2 e Dectina-1 (em adição à mediadores inflamatórios como IFN-γ e TNF-α) (LAN; WU; SUN; YANG *et al.*, 2018). Como resultado da interação entre PAMPs e PRRs várias cascatas de sinalização são iniciadas a fim de levar macrófagos à fagocitose, produção de citocinas e quimiocinas, produção de espécies reativas de oxigênio (EROs) e de nitrogênio (ERN) (PATIN; THOMPSON; ORR, 2019), e eliminação do patógeno invasor.

A fagocitose é um mecanismo ativo, dependente de energia, em que células do sistema imune (como neutrófilos, macrófagos, células dendríticas) reconhecem e ingerem partículas, microrganismos ou células apoptóticas em vesículas derivadas de membrana, chamada de “fagossomos”, que posteriormente, se fundirá a lisossomos formando o “fagolisossomo”, uma organela microbicida que devido ao baixo pH, presença de enzimas hidrolíticas (como proteases, lisozimas, lípases) e componentes do sistema NADPH oxidase, resulta na eliminação do componente internalizado (ABBAS; LITCHMAN; PILLAI, 2011; ROSALES; URIBE-QUEROL, 2017). Os macrófagos exercem essa função com maestria. É demonstrado que cerca de 15-30 min após exposição de camundongos à conídios de *A. flavus*, é possível identificar macrófagos alveolares com conídios internalizados em fagossomos, com aparentes agregados lisossomais próximos à essa estrutura. Em adição, os conídios fagocitados pelos macrófagos alveolares não apresentam sinais de germinação, demonstrando a efetividade destes fagócitos mononucleares contra conídios de *A. flavus* (MERKOW; EPSTEIN; SIDRANSKY; VERNEY et al., 1971).

A eliminação dos conídios pelos macrófagos é vista cerca de 120 min após o processo de fagocitose, *in vitro* (PERKHOFER; SPETH; DIERICH; LASS-FLORL, 2007). Dentre os mecanismos empregados para a eliminação do patógeno fagocitado, estão a produção de ERN (principalmente óxido nítrico (NO) através da ação da enzima óxido nítrico sintase indutível - iNOS), e produção de EROs (através da enzima Nicotinamida Adenina Dinucleotídeo Fosfato (NADPH) Oxidase), que

devido a ação de danos ao DNA, modificações pós-traducionais de proteínas e peroxiação lipídica, causam danos celulares ao patógeno invasor, culminando em sua eliminação (ABBAS; LITCHMAN; PILLAI, 2011; WARRIS; BALLOU, 2019).

Concomitantemente, a sinalização iniciada pela ação dos PRRs também resulta na produção de diversas citocinas e quimiocinas pelos macrófagos, como interleucina (IL)-1β, fator de necrose tumoral (TNF)-α, proteína inflamatória de macrófago (MIP)-1α, proteína quimioatraente de monócito (MCP)-1, dentre outras (PARK; MEHRAD, 2009; PHADKE; MEHRAD, 2005). O resultado dessa secreção é o recrutamento e ativação de células imunes da circulação, como neutrófilos, monócitos e linfócitos que também auxiliarão na eliminação fúngica (VAROL; MILDNER; JUNG, 2015).

Em resposta a esta gama de mediadores secretados pelas células do sistema imune, os macrófagos em decorrência de sua plasticidade, podem adquirir perfis fenotípicos distintos em resposta a condições do microambiente (TAN; WANG; LI; HONG *et al.*, 2016). Os fenótipos clássicos são M1 e M2. O perfil M1 é caracterizado por sua potente atividade microbicida e anti-tumoral, possuindo marcadores clássicos como a expressão de iNOS, óxido nítrico sintase 2, quimiocinas CXCL9, CXCL10, CXCL11, IL-12 e supressor de sinalização de citocina 3 (SOCS3) (LEOPOLD WAGER; WORMLEY, 2014). Macrófagos M1 são tipicamente induzidos por citocinas de padrão Th1 (como TNF-α, IFN-γ) ou ligantes de TLR (como lipopolissacarídeo (LPS)) (MURRAY, 2017), produzem EROs e ERNs, citocinas inflamatórias (como TNF-α, IL-1β, IL-6) e baixos níveis de IL-10 (SHAPOURI-MOGHADDAM, A.; MOHAMMADIAN, S.; VAZINI, H.; TAGHADOSI, M. *et al.*, 2018). Como consequência da resposta altamente inflamatória ditada por este tipo de macrófago, danos teciduais podem ocorrer com mecanismos de regeneração tecidual sendo afetados, contribuindo para o estabelecimento de uma inflamação crônica (SHAPOURI-MOGHADDAM, ABBAS; MOHAMMADIAN, SAEED; VAZINI, HOSSEIN; TAGHADOSI, MAHDI *et al.*, 2018). Em resposta a este processo, citocinas anti-inflamatórias como IL-4 e IL-13 são produzidas, estimulando a polarização dos macrófagos para um perfil M2, que contribuem para supressão e regulação do processo inflamatório, além da contribuição em processos de cicatrização de feridas (LEOPOLD WAGER; WORMLEY, 2014). A expressão de

arginase-1 (arg-1), encontrada na zona inflamatória 1 (FIZZ1), moléculas *like* quitinase (YM1 e YM2) são consideradas marcadores clássicos para macrófagos M2 (LEOPOLD WAGER; WORMLEY, 2014). Estudos utilizando macrófagos humanos demonstram que conídios do *A. fumigatus* são capazes de estimular uma polarização para perfil M1 (ZHANG; HE; GAO; WEI *et al.*, 2019). Em contrapartida, logo após a infecção de macrófagos alveolares murinos com conídios de *A. fumigatus*, a expressão de arg-1, YM1, CD206 é detectada, demonstrando uma polarização para o perfil M2, tendo estas células, papel protetor contra a infecção (BHATIA; FEI; YARLAGADDA; QI *et al.*, 2011). As informações sobre o perfil de polarização de macrófagos frente infecção pelo *A. flavus* são escassos, demonstrando a necessidade de mais estudos, considerando a importância dos macrófagos para a resolução da infecção.

1.3 VESÍCULAS EXTRACELULARES

Secretadas por eucariontes e procariontes no ambiente extracelular (RYBAK; ROBATZEK, 2019), vesículas extracelulares (EVs) são estruturas esféricas, circundadas por bicamada lipídica, de tamanho variável (entre 20-2.000 nm), que carreiam diversos componentes, como proteínas, lipídios, ácidos nucleicos e polissacarídeos (JOFFE; NIMRICHTER; RODRIGUES; DEL POETA, 2016; SCHOREY; CHENG; SINGH; SMITH, 2015). De acordo com sua biogênese, EVs são classificadas como microvesículas, exossomos e corpos apoptóticos (YANEZ-MO; SILJANDER; ANDREU; ZAVEC *et al.*, 2015). As microvesículas são produzidas a partir do brotamento da membrana plasmática, possuindo tamanho variando de 50-100 nm (RODRIGUES; FAN; LYON; WAN *et al.*, 2018). Já os exossomos (20-100 nm) são produzidos através do sistema endocítico da célula, sendo liberados ao meio extracelular através da fusão dos corpos multicelulares com a membrana plasmática (SCHOREY; CHENG; SINGH; SMITH, 2015; YANEZ-MO; SILJANDER; ANDREU; ZAVEC *et al.*, 2015). Por fim, os corpos apoptóticos são as maiores vesículas, possuindo tamanho de 500-2000 nm, produzidas em decorrência da fragmentação celular ocorrida durante o processo de apoptose (SAMANTA; RAJASINGH; DROSOS; ZHOU *et al.*, 2018). É importante destacar que a literatura

sobre EVs produzidas por fungos não permite a diferenciação entre os exossomos, microvesículas ou corpos apoptóticos (OLIVEIRA; RIZZO; JOFFE; GODINHO *et al.*, 2013), e o termo “vesículas extracelulares” se refere aos compartimentos circundados por membrana lipídica que compreendem tamanhos entre 30-400 nm (OLIVEIRA; RIZZO; JOFFE; GODINHO *et al.*, 2013).

A importância das EVs é refletida pela sua capacidade de carrear importantes biomoléculas, sendo reconhecidas por seu importante papel, principalmente na manutenção da homeostase e comunicação celular (SAMANTA; RAJASINGH; DROSOS; ZHOU *et al.*, 2018; YANEZ-MO; SILJANDER; ANDREU; ZAVEC *et al.*, 2015). Porém, EVs também são produzidas em condições patológicas como câncer, doenças neurodegenerativas (como Parkinson, Alzheimer e esclerose múltipla), doenças autoimunes (artrite reumatóide), obesidade e durante infecções (seja por vírus, bactéria, protozoário ou fungos), exercendo papel modulador na fisiopatologia da doença (FREITAS; BONATO; PESSONI; RODRIGUES *et al.*, 2019; WOITH; FUHRMANN; MELZIG, 2019).

A produção de EVs por fungos foi descrita pela primeira vez em 2007, por Rodrigues e colaboradores, onde demonstraram que *Cryptococcus neoformans* secretam vesículas dotadas com glucoronoxilomanana (GXM) (RODRIGUES; NIMRICHTER; OLIVEIRA; FRASES et al., 2007). O GXM é um polissacarídeo que compreende cerca de 90% da cápsula do *C. neoformans*, sendo considerado o principal fator de virulência deste fungo, durante o estabelecimento da infecção (ZARAGOZA; RODRIGUES; DE JESUS; FRASES *et al.*, 2009). Em adição ao GXM, outros fatores de virulência foram identificados no interior das EVs (lacase, urease, fosfatase ácida, proteínas antioxidantes, dentre outros), demonstrando que as vesículas podem ser consideradas “bolsas de virulência”, um mecanismo em que o fungo se aproveita da compartimentalização destes fatores, para que possam alcançar o ambiente extracelular, modulando a interação fungo-hospedeiro (RODRIGUES; NAKAYASU; OLIVEIRA; NIMRICHTER *et al.*, 2008).

Em adição ao *C. neoformans*, diversas outras espécies fúngicas se mostraram produtoras de EVs: *Histoplasma capsulatum, Candida albicans, C. parapsilosis, Sporothrix schenckii* (ALBUQUERQUE; NAKAYASU; RODRIGUES;

FRASES et al., 2008), *Saccharomyces cerevisiae* (OLIVEIRA; NAKAYASU; JOFFE; GUIMARÃES et al., 2010), *Paracoccidioides brasiliensis* (VALLEJO; MATSUO; GANIKO; MEDEIROS et al., 2011), *Malassezia sympodialis* (GEHRMANN; QAZI; JOHANSSON; HULTENBY et al., 2011), *Alternaria infectoria* (SILVA; PRADOS-ROSALES; ESPADAS-MORENO; WOLF *et al.*, 2014), *C. gatii* (BIELSKA; SISQUELLA; ALDEIEG; BIRCH et al., 2018), *S. brasiliensis* (IKEDA; DE ALMEIDA; JANNUZZI; CRONEMBERGER-ANDRADE et al., 2018), *Pichia fermentans* (LEONE; BELLANI; MUCCIFORA; GIORGETTI et al., 2018), *Trichophyton interdigitale* (BITENCOURT; REZENDE; QUARESEMIN; MORENO et al., 2018), *Trichoderma reesei* (DE PAULA; ANTONIETO; NOGUEIRA; RIBEIRO et al., 2019), *Fusarium oxysporum* f. sp. *vasinfectum* (BLEACKLEY; SAMUEL; GARCIA-CERON; MCKENNA et al., 2019), *A. fumigatus* (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO et al., 2019), dentre outras.

Apesar dos diversos estudos mostrando a produção das EVs entre as mais diferentes espécies fúngicas, os mecanismos de biogênese das EVs não são completamente elucidados. Rodrigues *et al,* 2013, sugerem a origem a partir de frações citoplasmáticas que resultam do remodelamento da membrana plasmática em um mecanismo chamado de “macropinocitose invertida” (RODRIGUES; FRANZEN; NIMRICHTER; MIRANDA, 2013). Wolf *et* al, 2014, demonstraram que vesículas podem ser secretadas isoladamente (se originando da membrana plasmática), ou em múltiplos eventos que aparentemente são resultado da fusão dos corpos multivesiculares com membrana plasmática, liberando essas estruturas entre o espaço da membrana e parede celular (WOLF; ESPADAS-MORENO; LUQUE-GARCIA; CASADEVALL, 2014). Análises alterando expressão de genes relacionados com via endocítica (mais especificamente *snf7*) e também do sistema de secreção via complexo de Golgi (GRASP e *SEC4*), não forneceram resultados conclusivos. As cepas mutantes para os genes citados acima, apresentaram a produção de vesículas com conteúdos modificados, porém, sem modificações na secreção destas estruturas (OLIVEIRA; NAKAYASU; JOFFE; GUIMARÃES *et al.*, 2010). Estes dados demonstram que as EVs produzidas por fungos podem possuir origens variadas, sendo esta informação refletida na heterogeneidade de composição das vesículas. Este fato reforça a necessidade de mais estudos no

aspecto de aprimoramento de protocolos para a possibilidade de separação das diferentes populações de EVs e descrição do seu conteúdo, com objetivo de identificar marcadores que auxiliem no esclarecimento da origem das diferentes populações (DE TOLEDO MARTINS; SZWARC; GOLDENBERG; ALVES, 2019; RODRIGUES; CASADEVALL, 2018; RODRIGUES; GODINHO; ZAMITH-MIRANDA; NIMRICHTER, 2015).

1.3.1 Vesículas extracelulares fúngicas e resposta imune

As EVs produzidas por fungos se mostram importantes moduladores da interação patógeno-hopedeiro, podendo participar de mecanismos anti-microbicidas exercidos pelas células imunológicas do hospedeiro (BIELSKA; MAY, 2019; RYBAK; ROBATZEK, 2019).

O primeiro estudo que demonstrou capacidade imunomoduladora de EVs produzidas por fungos foi em modelo de *M. sympodialis*, onde Gehrmann e colaboradores demonstraram que as vesículas carreavam alérgenos e epítopos que desencadeiam uma resposta de produção de citocinas (IL-4 e TNF-α) em células mononucleares do sangue periférico (GEHRMANN; QAZI; JOHANSSON; HULTENBY *et al.*, 2011). Em adição, EVs produzidas pelo *M. sympodialis* são internalizadas por queratinócitos e monócitos (JOHANSSON; VALLHOV; HOLM; GEHRMANN *et al.*, 2018), demonstrando que essas vesículas interagem com células do sistema imune inato e também, da pele, podendo exercer função na patogênese do eczema atópico, doença de pele inflamatória em que o *M. sympodialis* tem forte associação (FREITAS; BONATO; PESSONI; RODRIGUES *et al.*, 2019; JOHANSSON; VALLHOV; HOLM; GEHRMANN *et al.*, 2018).

EVs produzidas por *C. neoformans* também são internalizadas por célula do sistema imune do hospedeiro, mais especificamente, macrófagos. Além da fagocitose, EVs estimulam a secreção de TNF-α, IL-10, fator de crescimento transformante (TGF)-β, NO, além de estimular a capacidade microbicida destes fagócitos, sugerindo um efeito protetor dessas estruturas em condições de criptococose (OLIVEIRA; FREIRE-DE-LIMA; NOSANCHUK; CASADEVALL *et al.*,

2010). É interessante notar, que além de ações em células da imunidade inata, é provável que EVs produzidas por *C. neofomans* possam ter algum papel no desenvolvimento da imunidade adaptativa, visto que anticorpos presentes no soro de paciente com criptococose possuem reatividade contra proteínas pertencentes às EVs de *C. neoformans* (RODRIGUES; NAKAYASU; OLIVEIRA; NIMRICHTER *et al.*, 2008).

Similarmente, proteínas vesiculares de *H. capsulatum* também são reconhecidas por soro de pacientes com histoplasmose (ALBUQUERQUE; NAKAYASU; RODRIGUES; FRASES *et al.*, 2008). Em contrapartida, EVs produzidas por este fungo inibem a atividade fagocítica e microbicida de macrófagos derivados da medula óssea (BMDM) (BALTAZAR; ZAMITH-MIRANDA; BURNET; CHOI *et al.*, 2018), demonstrando que estas estruturas possuem atividade mais supressora do que protetora em condições de infecção por *H. capsulatum* (FREITAS; BONATO; PESSONI; RODRIGUES et al., 2019). Tal efeito supressor também é visto quando EVs de *S. brasiliensis* são inoculadas em camundongos BALB/c, previamente à infecção subcutânea com este fungo. Como resultado, maior diâmetro da ferida em associação com maior carga fúngica é observado nos camundongos tratados com EVs, demonstrando que as essas estruturas podem auxiliar no estabelecimento da infecção por *S. brasiliensis* (IKEDA; DE ALMEIDA; JANNUZZI; CRONEMBERGER-ANDRADE et al., 2018).

Contrariamente, o tratamento de larvas (*Galleria mellonella*) com EVs produzidas por *C. albicans*, previamente à infecção, resultou em maior atividade microbicida destas, sugerindo um papel protetor das EVs. Em adição, macrófagos e células dendríticas são capazes de fagocitar EVs e estimular a produção de mediadores como NO, IL-12, TNF-α, TGF-β e IL-10. Além da produção de mediadores, a estimulação de células dendríticas com EVs, resultou na maior expressão de moléculas co-estimulatórias como CD86 e complexo principal de histocompatibilidade (MHC) de classe II, sugerindo que EVs estão relacionadas com ambas as respostas imune inata e adaptativa, frente a quadros de candidíase (VARGAS; ROCHA; OLIVEIRA; ALBUQUERQUE *et al.*, 2015).

Além de mecanismo efetores de macrófagos (como fagocitose, produção de citocinas, NO, EROs), a relação entre polarização e EVs foi demonstrada em *P. brasiliensis.* Em 2016, da Silva e colaboradores descreveram que EVs produzidas por *P. brasiliensis*, são capazes de estimular a produção de mediadores inflamatórios (como NO, TNF-α, IL-6, IL-12p40, IL-12p70, IL-1α, IL-1β), aumentar a atividade fungicida de macrófagos peritoneais murinos, induzir uma polarização para perfil M1, e induzir também, a troca de perfis M2 para M1 (DA SILVA; ROQUE-BARREIRA; CASADEVALL; ALMEIDA, 2016). Similarmente, Bitencourt e colaboradores, também demonstraram a polarização de macrófagos para perfil M1 em resposta à EVs produzidas por *T. interdigitale*, sendo este mecanismo dependente de TLR2 (BITENCOURT; REZENDE; QUARESEMIN; MORENO *et al.*, 2018).

No que diz respeito a espécies de *Aspergillus*, até o presente momento, a produção de EVs foi descrita apenas para *A. fumigatus.* Além da produção e caracterização, Souza e colaboradores demonstraram que macrófagos pré-estimulados com EVs produzidas por *A. fumigatus*, possuem maior capacidade fagocítica e produzem mediadores inflamatórios como TNF-α e CCL2 (esse efeito de produção de citocinas é reforçado quando as células estimuladas são desafiadas com conídios do *A. fumigatus*). Similarmente, neutrófilos murinos derivados da medula óssea, também apresentaram maior atividade fagocítica e microbicida, produção de mediadores como TNF-α e IL-1β em resposta à estimulação com EVs e previamente ao desafio com conídios de *A. fumigatus,* demonstrando que EVs de *A. fumigatus* são bioativas, podendo auxiliar no controle da infecção (SOUZA; BALTAZAR; CARREGAL; GOUVEIA-EUFRASIO *et al.*, 2019).

2. Objetivos

2.1 OBJETIVO GERAL

Avaliar a produção de vesículas extracelulares por *A. flavus*, assim como, se são estruturas bioativas com capacidade de estimular resposta imunológica em macrófagos *in vitro.* Em adição, em modelos *in vivo* de *G. mellonella*, avaliar a influência das EVs sob a sobrevivência das larvas.

2.2 OBJETIVOS ESPECÍFICOS

1. Avaliar a produção de EVs por *A. flavus;*
2. Caracterizar as EVs por *Nanoparticle-Tracking Analysis* (NTA);
3. Analisar os efeitos *in vitro* das EVs em macrófagos derivados da medula óssea, quanto aos parâmetros:

3.1. Produção de citocinas: TNF-α, IL-6, IL-1β e IL-10;

3.2. Produção de óxido nítrico;

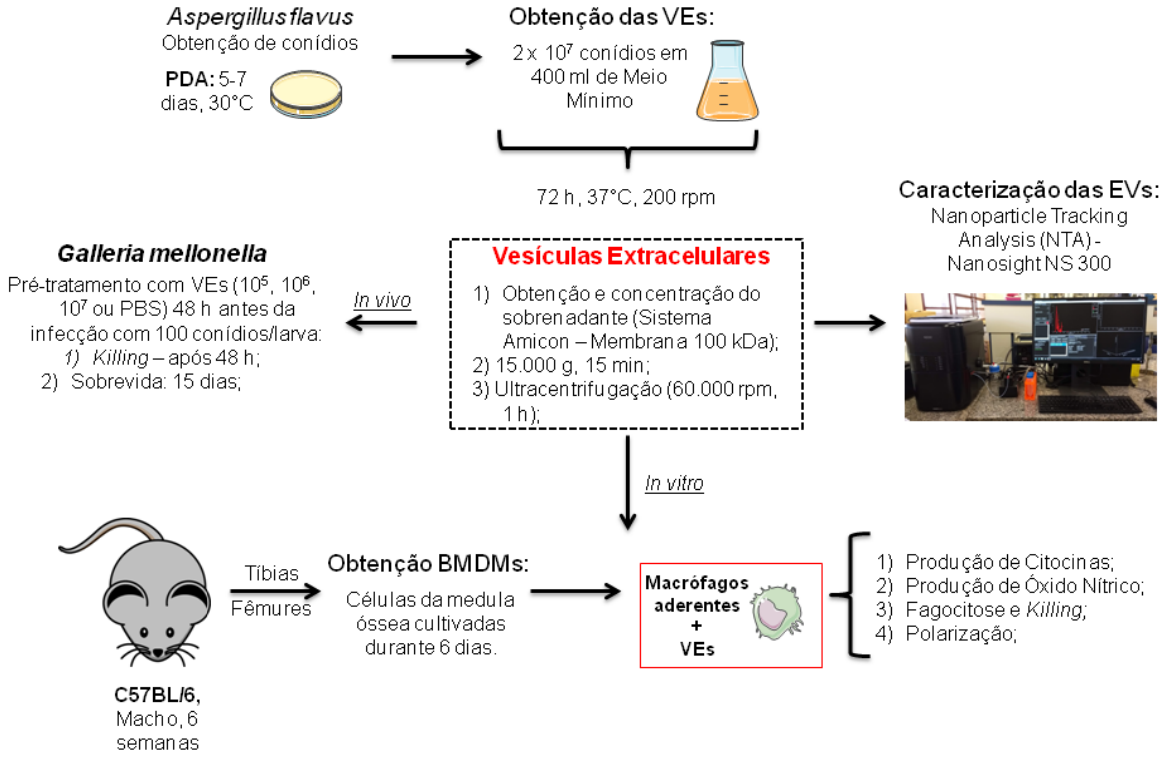
3.3. Atividade fagocítica e microbicida;

3.4. Perfil de polarização para fenótipos M1 ou M2;

4. Analisar a atividade *in vivo* das EVs, utilizando larvas de *G. mellonella* avaliando sobrevivência e atividade microbicida;

3. Delineamento Experimental

Figura 2 – Delineamento Experimental



Legenda: Para produção de conídios, *A. flavus* foi cultivado em PDA (*Potato Dextrose Agar*) por 5-7 dias, 30ºC. Conídios foram coletados e quantificados com auxílio de câmara de Neubauer. Um total de $2x10^7$ conídios foram inoculados em 400 ml de meio mínimo, permanecendo 72 h em cultura a 37°C, 200 rpm. A partir do sobrenadante desta cultura, as vesículas extracelulares foram isoladas, e posteriormente caracterizadas por *Nanoparticle Tracking Analysis*. A capacidade imunogênica das EVs foi avaliada adicionado-as em culturas de BMDMs, e avaliando respostas efetoras de macrófagos, como: produção de citocinas e óxido nítrico, atividade fagocítica e microbicida, perfil de polarização para M1 ou M2. Em modelos *in vivo*, larvas de *G. mellonella* foram estimuladas com EVs (10^5, 10^6, 10^7 ou PBS) previamente ao desafio com conídios de *A. flavus* (100 conídios/larva). Análises de sobrevida e CFU foram realizadas para analisar possível papel protetor das EVs durante a infecção por *A. flavus.*

Capítulo II

Manuscrito: BRAUER, V. S.; PESSONI, A. M; BITENCOURT, T. A.; de PAULA, R. G.; ROCHA, L. O.; GOLDMAN, G. H.; ALMEIDA, F. Extracellular Vesicles from *Aspergillus flavus* Induce M1 Polarization *In Vitro*. **mSphere**, 5, n. 3, May 6, 2020.

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RESEARCH ARTICLE
Host-Microbe Biology

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-1β. 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-

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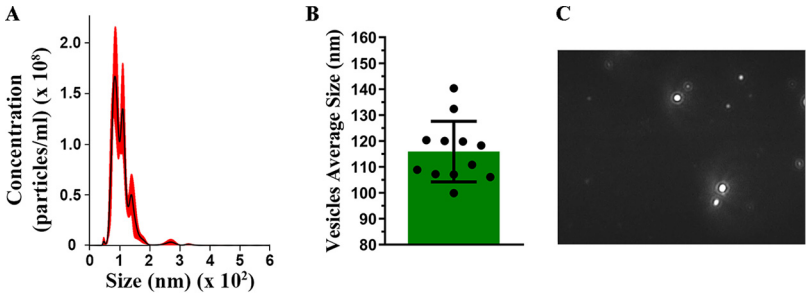

FIG 1 Extracellular vesicles (EVs) produced by *Aspergillus flavus*. Nanoparticle-tracking analysis of EVs isolated from *A. flavus* culture supernatant was performed using NanoSight NS300. (A) Representative histogram depicting the particle-size distribution and concentration of EV profiles from *A. flavus* (EVs × 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 μg/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

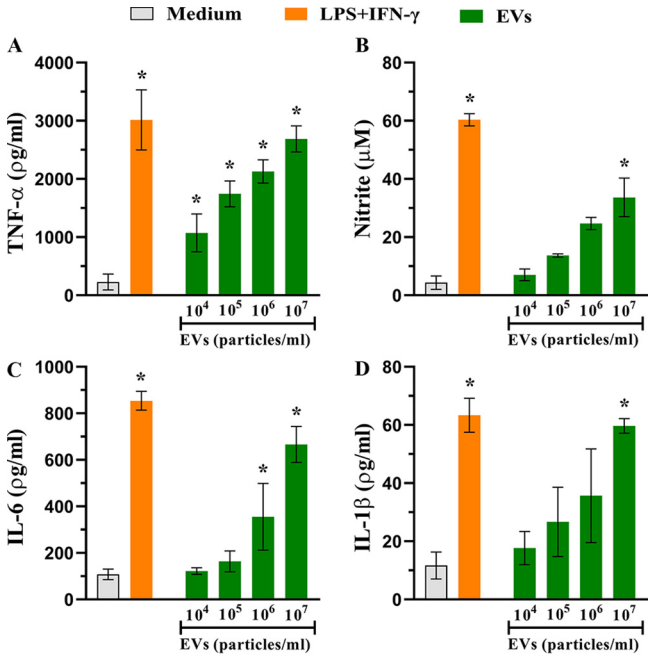

FIG 2 EVs from *A. flavus* induce the production of inflammatory mediators by bone marrow-derived macrophages (BMDMs). BMDMs obtained from C57BL/6 mice were cultured with different concentrations of EVs (10^4 to 10^7 particles/ml) for 48 h at 37°C. As positive and negative controls, the BMDMs were treated 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

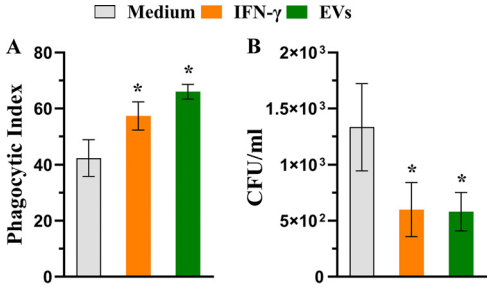

FIG 3 *A. flavus* EVs stimulate microbicidal activity of BMDMs. (A) BMDMs were plated on glass coverslips and cultured with EVs (10^7 particles/ml) for 30 min and were treated with *A. flavus* conidia (macrophages/conidia = 1:1) for 4 h at 37°C, and the phagocytic index was determined. (B) BMDM previously treated with EVs (10^7 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. *, $P < 0.05$.

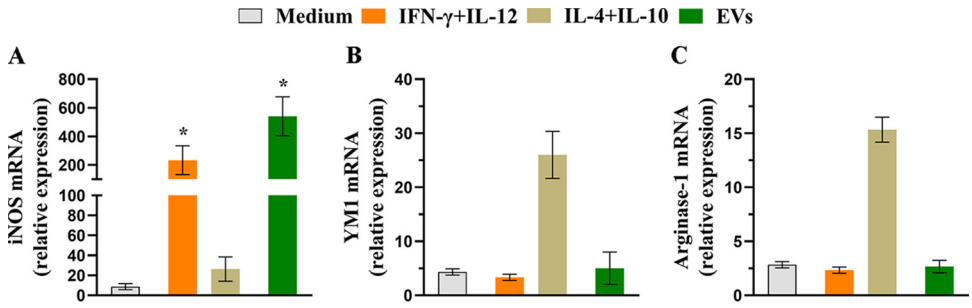

FIG 4 EVs induce M1 polarization of BMDMs. BMDMs were cultured with 10^7 particles/ml for 6 h. The cells were further treated with IFN-γ (2 ng/ml) plus IL-12p40 (50 ng/ml) as a positive control for M1 phenotype or with IL-4 (50 ng/ml) plus IL-10 (50 ng/ml) as a positive control of M2 phenotype or with 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

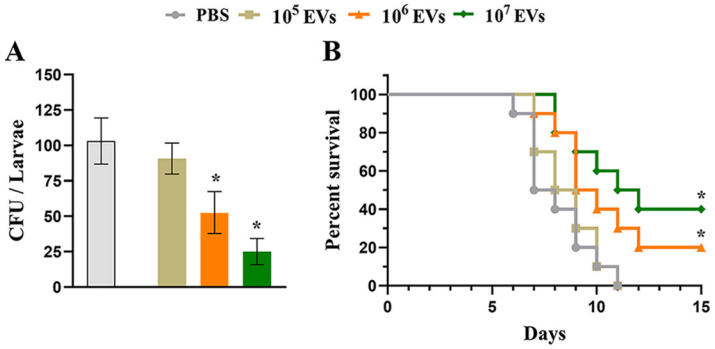

FIG 5 *A. flavus* EVs induce a protective effect on *Galleria mellonella* in an *in vivo* model. (A) *G. mellonella* larvae ($n = 5$ per group) were stimulated with *A. flavus* EVs (10^5, 10^6, and 10^7 particles) or with PBS as a control 48 h prior to infection with *A. flavus* conidia. Larvae were 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 biological 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 adaptative 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× salt solution [120 g/liter $NaNO_3$, 10.4 g/liter KCl, 30 g/liter KH_2PO_4, 10.4 g/liter $MgSO_4 \cdot 7H_2O$], 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 $(NH_4)_2MoO_4$, 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 × *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 μg/ml) plus gamma interferon (IFN-γ) (2 ng/ml) or IFN-γ (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 micomoles).

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-1β, 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 IFN-γ (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 ± 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 ± 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.

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Capítulo III – Considerações Finais

Conclusões

Conclusões

Demonstramos que *A. flavus* secreta vesículas extracelulares biologicamente ativas. As EVs foram capazes de estimular macrófagos a produzir importantes mediadores inflamatórios, aumentar capacidade fagocítica e microbicida, caracterizando um perfil de polarização M1. Estes dados fornecem grande contribuição para o entendimento dos mecanismos imunológicos frente à infecção por *A. flavus*, visto que informações sobre as formas de combate à infecção pelo hospedeiro são escassas. O resultado da ação inflamatória dos macrófagos é refletido em modelo *in vivo*, onde as EV estimularam a maior eliminação do fungo e sobrevivência do hospedeiro, no caso, larvas de *G. mellonella.*

O presente trabalho, além de nos fornecer importantes indícios sobre os mecanismos de infecção exercidos por *A. flavus*, nos possibilita um maior entendimento de como o sistema imune do hospedeiro lida com esse patógeno invasor. Essa nova descrição de EVs nos permite inferir sobre um possível alvo terapêutico contra aspergilose, visto que essas estruturas se mostraram imunogênicas, auxiliando na eliminação fúngica e controle da infecção por *A. flavus.*

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Anexos

Anexo I

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Review

Antifungal Agents in Agriculture: Friends and Foes of Public Health

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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 flucocytosine 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].

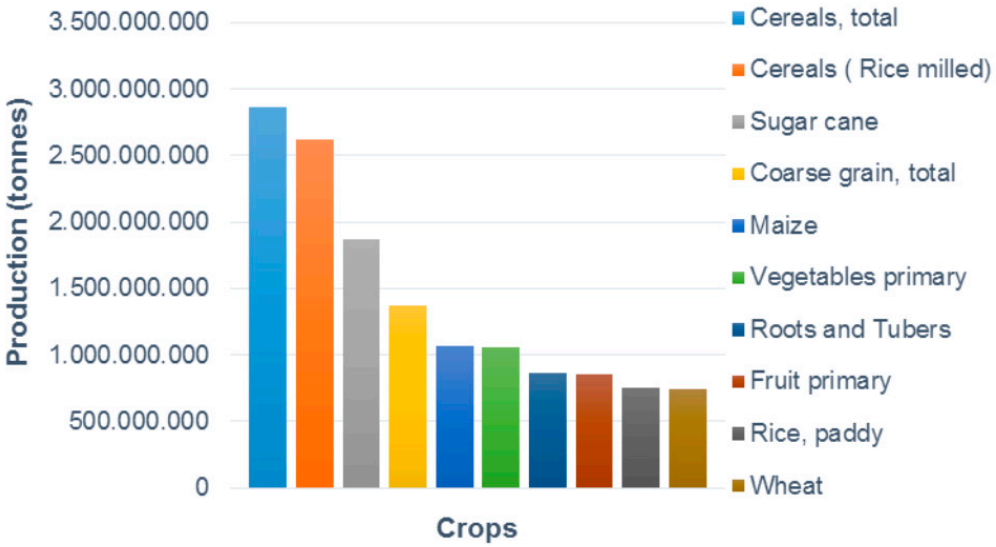

Figure 1. The annual global production of major crops during 2013–2017 (Source: FAOSTAT. www.fao.org. Access on: 06 November 2018).

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

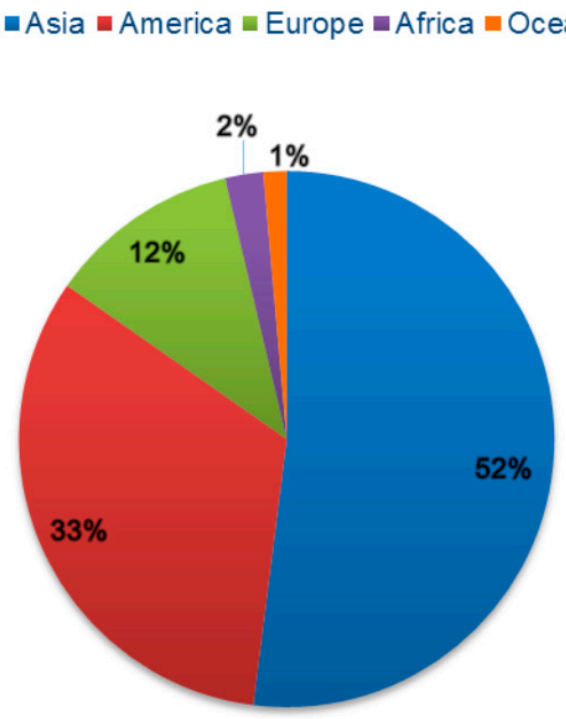

Figure 2. Use of pesticides in the world during 2012–2016 (Source: FAOSTAT. www.fao.org. Access on: 17 December 2018).

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]	*Botrytis cinerea*, *Venturia inaequalis* [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, fluxapryroxad, fluxypyram, thifluzamide, furametpyr [59]	*Botrytis cinerea*, *Alternaria alternate*, *Didymella brioniae*, *Podosphaeera xanthii*, *Corynespora cassiicola* [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]	*Botrytis cinerea*, *Venturia inaequalis*, *Oculimacula* 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]	*Erysiphe necator*, *Pseudopernospora cubensis*, *Venturia inaequalis*, *Alternaria solani*, *Pyrenophora teres*, *Pythium aphanidermatum*, *Pyrenophora tritici-repentis* [65]	Point mutations in the mitochondrial cytochrome b (*cyt b*) gene (G143A, F129L, G137R) [57,70]
Morpholine	Inhibition of ergosterol synthesis by blocking Δ14-reductase and Δ8-Δ7-isomerase [6]	Aldomorph, fenpropimorph, dodemorph, tridemorph [65]	Decreased sensitivity in powdery mildews [16]	Unknown [16]
Azole	Suppression of ergosterol synthesis by inhibiting 14α-demethylase [12]	Imazalil, oxpoconazole, triflumizole, diniconazole, epoxiconazole, flutriafol [65]	*Zymoseptoria tritici*, *Venturia inaequalis*, *Penicillium digitatum*, *Cercospora beticola*, *Monilinia fructicola*, *Brumeriela jaapii*, *Botrytis cinérea*, *Penicillium digitatum*, *Zymoseptoria tritici* [71,72]	Mutations in *cyp51*, upregulation of *cyp51* 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, fluxapryroxad, and fluxypyram, 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*, *Podosphaeera 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 bezylcarbamates [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 bc_1 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*, *Pseudopernospora 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: Δ14-reductase and Δ8-Δ7-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 14α-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., *Thrichosporon 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 TR_{34}/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* (14α-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 (TR_{46}/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 TR_{34}/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 (C60), 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]	*Eucalyptus grandis* [140]	Scleroderma A and B Triterpenoid lanostane [140]	*Candida albicans* *C. tropicalis* *C. grusei* *C. parapsiosis* [140]
Xylaria spp. [141]	*Azadirachta indica* [127,142]	Bioactive compounds [141]	*C. albicans* *Aspergillus niger* *Fusarium avenaceum* [127,142]
Mycosphaerella spp. [143]	*Eugenia bimarginata* [143]	Eicosanoid acids [143]	*Cryptococcus neoformans* *C. gattii* [143]
Cryptosporiopsis quercina [144]	Wood species in Europe [144]	Cryptocandin lipopeptide [144]	*Botrytis cinerea* [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.

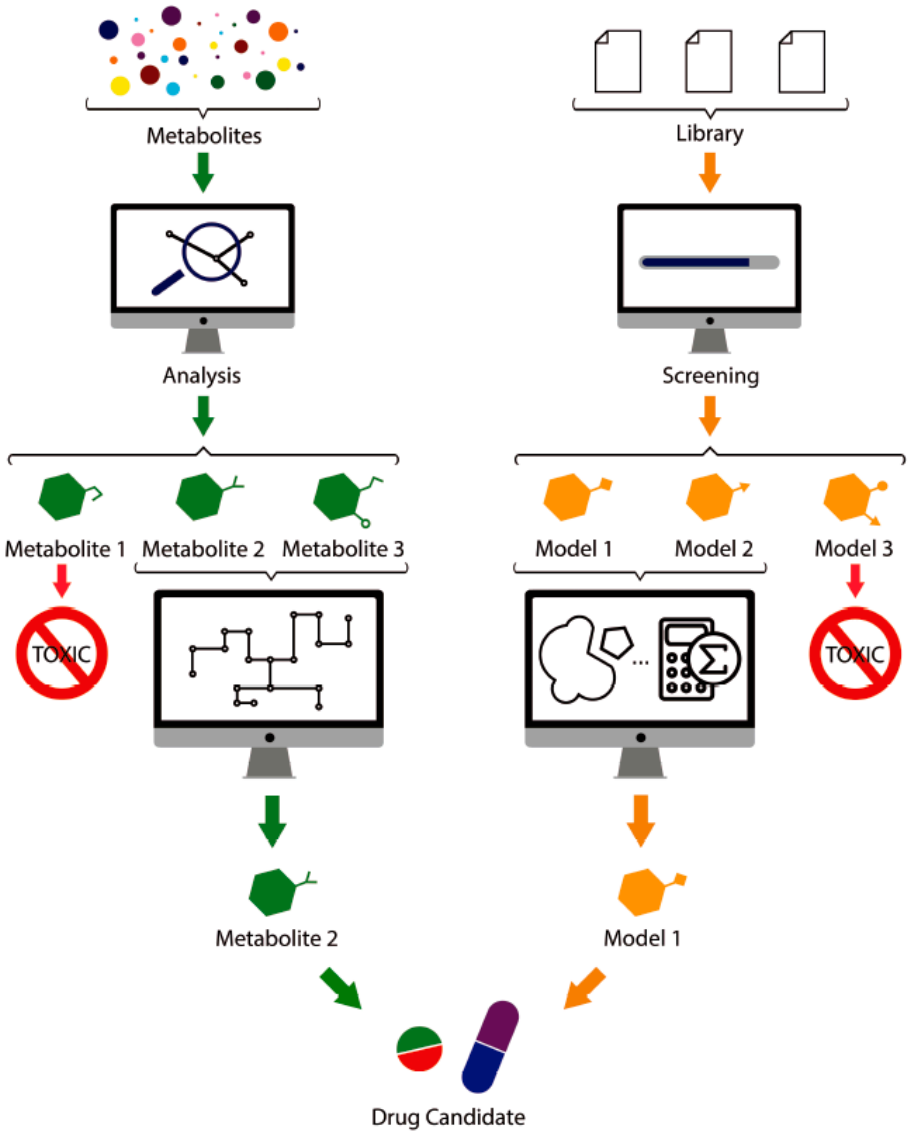

Figure 3. The use of metagenomics in the development of new drug candidates. The left path illustrates metabolite evaluation to identify bioactive or toxic molecules overlaid with metabolic pathways and the selected drug candidate. The right path illustrates the molecular library screening used to generate 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.

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Anexo II

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RESEARCH ARTICLE
Molecular Biology and Physiology

Functional Characterization of Clinical Isolates of the Opportunistic Fungal Pathogen *Aspergillus nidulans*

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ABSTRACT *Aspergillus nidulans* is an opportunistic fungal pathogen in patients with immunodeficiency, and virulence of *A. nidulans* isolates has mainly been studied in the context of chronic granulomatous disease (CGD), with characterization of clinical isolates obtained from non-CGD patients remaining elusive. This study therefore carried out a detailed biological characterization of two *A. nidulans* clinical isolates (CIs), obtained from a patient with breast carcinoma and pneumonia and from a patient with cystic fibrosis that underwent lung transplantation, and compared them to the reference, nonclinical FGSC A4 strain. Both CIs presented increased growth in comparison to that of the reference strain in the presence of physiologically relevant carbon sources. Metabolomic analyses showed that the three strains are metabolically very different from each other in these carbon sources. Furthermore, the CIs were highly susceptible to cell wall-perturbing agents but not to other physiologically relevant stresses. Genome analyses identified several frameshift variants in genes encoding cell wall integrity (CWI) signaling components. Significant differences in CWI signaling were confirmed by Western blotting among the three strains. *In vivo* virulence studies using several different models revealed that strain MO80069 had significantly higher virulence in hosts with impaired neutrophil function than the other strains. In summary, this study presents detailed biological characterization of two *A. nidulans sensu stricto* clinical isolates. Just as in *Aspergillus fumigatus*, strain heterogeneity exists in *A. nidulans* clinical strains that can define virulence traits. Further studies are required to fully characterize *A. nidulans* strain-specific virulence traits and pathogenicity.

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IMPORTANCE Immunocompromised patients are susceptible to infections with opportunistic filamentous fungi from the genus *Aspergillus*. Although *A. fumigatus* is the main etiological agent of *Aspergillus* species-related infections, other species, such as *A. nidulans*, are prevalent in a condition-specific manner. *A. nidulans* is a predominant infective agent in patients suffering from chronic granulomatous disease (CGD). *A. nidulans* isolates have mainly been studied in the context of CGD although infection with *A. nidulans* also occurs in non-CGD patients. This study carried out a detailed biological characterization of two non-CGD *A. nidulans* clinical isolates and compared the results to those with a reference strain. Phenotypic, metabolomic, and genomic analyses highlight fundamental differences in carbon source utilization, stress responses, and maintenance of cell wall integrity among the strains. One clinical strain had increased virulence in models with impaired neutrophil function. Just as in *A. fumigatus*, strain heterogeneity exists in *A. nidulans* clinical strains that can define virulence traits.

KEYWORDS *Aspergillus nidulans*, clinical isolates, genome sequencing, metabolomics

Fungal pathogen-related infections are now estimated to result in a higher number of human deaths than tuberculosis or malaria alone (1–3). The majority of systemic fungal infections are caused by *Candida* spp., *Pneumocystis* spp., *Cryptococcus* spp., and *Aspergillus* spp. (4, 5). Of the hundreds of known *Aspergillus* spp., only a few cause disease in animals, with the most prominent being *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger*, and *Aspergillus terreus* (6, 7).

The primary route of infection of *Aspergillus* spp. is via the inhalation of conidia (asexual spores). In immunocompetent individuals, inhaled conidia are rapidly cleared by pulmonary resident and recruited neutrophils and macrophages, together preventing the onset of infection (8–10). However, disturbances to the immune system may render an individual susceptible to infection by *Aspergillus* spp. (11). The severity of infection largely depends on fungal species and genotype, the host immunological status, and host lung structure (6). Invasive aspergillosis (IA) is the most severe disease caused by *Aspergillus* spp. and is characterized by systemic host invasion, resulting in high mortality rates (30 to 95%) (2, 10, 11).

Patient populations with a highest risk of IA are (i) those with prolonged neutropenia from intensive myeloablative chemotherapy, (ii) cancer patients who are immunosuppressed due to chemotherapy and/or radiotherapy, (iii) those with cystic fibrosis, a hereditary disease that affects the lungs, (iv) and those with genetic disorders resulting in primary immune deficiencies, such as chronic granulomatous disease (CGD) (12, 13). CGD is a genetic disorder that affects 1 in 250,000 people, and in ~80% of all cases subjects are of the male sex. CGD is caused by mutations in the genes encoding any of the five structural components of the NADPH-oxidase complex, an enzyme complex important for superoxide anion and downstream reactive oxygen species (ROS) production in phagocytic cells (14). As a result, immune cells are unable to efficiently kill microorganisms, and these microorganisms can then become pathogenic in such patients (13, 14)

Although *A. fumigatus* is the main etiological agent of *Aspergillus*-related infections in immunocompromised patients, other *Aspergillus* spp. have been found to have a high infection rate under some conditions. *A. nidulans* infections are not commonly reported in immunocompromised patients, except for subjects suffering from CGD (15, 16). In CGD patients, *A. fumigatus* and *A. nidulans* are responsible for 44% and 23%, respectively, of all fungal infections (15, 16). Infections with *A. nidulans* cause mortality in 27 to 32% of CGD patients (15), and in comparison to *A. fumigatus*, *A. nidulans* isolates have high virulence, invasiveness, dissemination, and resistance to antifungal drugs in these patients (17). Hence, *A. nidulans* infections have been studied mainly in the context of CGD although this fungal species can also be virulent in non-CGD, immunocompromised patients (18). In comparison to research on *A. fumigatus*, investigations into *A. nidulans* isolate virulence have been neglected, with very few studies

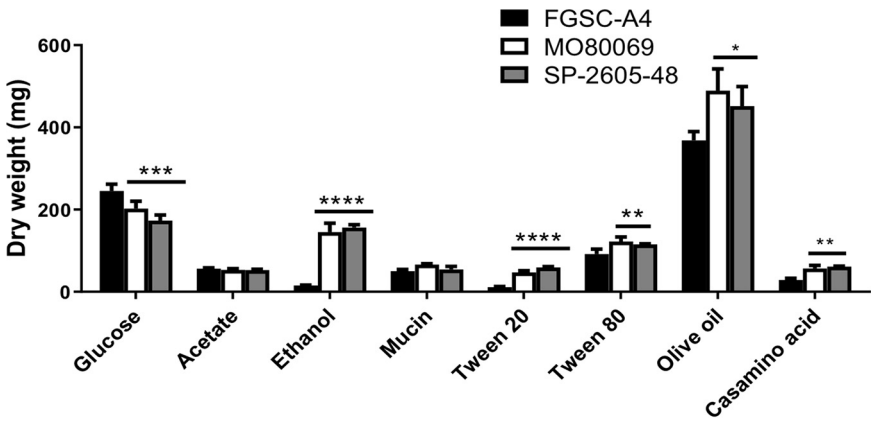

FIG 1 The *A. nidulans* clinical isolates exhibit improved growth in the presence of alternative carbon and lipid sources. Strains were grown in liquid MM supplemented with glucose, acetate, ethanol, mucin, Tween 20 and 80, olive oil, and Casamino Acids at 37°C for 48 h (glucose) or 72 h (others) before fungal biomass was freeze-dried and weighed. Standard deviations were determined from biological triplicates in a one-way ANOVA with Tukey's posttest comparing growth of the clinical isolates to that of the FGSC A4 reference strain (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

having investigated the genetic and metabolic features of *A. nidulans* clinical strains, isolated from CGD and non-CGD patients, in the context of stress responses encountered during human host infection as well as during interactions with host immune responses (18–21).

The aim of this work was to carry out a detailed molecular, phenotypic, and virulence characterization of two *A. nidulans* clinical isolates (CIs) from (i) a patient with breast carcinoma and pneumonia and (ii) a patient with cystic fibrosis who underwent lung transplantation and to compare the results to those with the well-characterized, wild-type isolate FGSC A4 (A4).

RESULTS

***A. nidulans* clinical isolates have increased growth in comparison to that of the reference strain in the presence of alternative carbon sources.** Fungal metabolic plasticity, which allows growth in unique and diverse ambient and host microenvironments, has long been hypothesized to contribute to *Aspergillus* virulence, with carbon sources such as glucose (22), ethanol (23), and acetate (24) being predicted to be actively used during *in vivo* infection. In addition, fatty acids and lipids are also thought to serve as major nutrient sources during mammalian host colonization, as is evident by the importance of key glyoxylate cycle enzymes in fungal virulence (25). We therefore characterized growth by determining the fungal dry weight of the two *A. nidulans* CIs in the presence of minimal medium (MM) supplemented with different physiologically relevant carbon sources, namely, glucose, acetate, ethanol, and lipids, and compared the results to those with the FGSC A4 reference strain. A significant reduction in growth was observed for both CIs in the presence of glucose, whereas both CIs had significantly increased growth in the presence of the alternative carbon sources ethanol, Casamino Acids, and the lipids Tween 20 (a source of lauric, palmitic, and myristic acids) (26), Tween 80 (which contains principally oleate) (26), and olive oil (triacylglycerols and free fatty acids) (27) (Fig. 1). In contrast, no difference in fungal biomass accumulation was observed in the presence of acetate and the lung-resident glycoprotein mucin (Fig. 1). These results suggest that the *A. nidulans* CIs have improved growth relative to that of the reference strain in the presence of most of the alternative carbon sources tested here, including different lipids.

Metabolic profiles differ among the *A. nidulans* clinical isolates and the reference strain in the presence of different carbon sources. To further investigate nutrient utilization in the *A. nidulans* CIs, the metabolic profiles of strains MO80069 and SP-2605-48 were determined and compared to the profile of the reference strain A4.

TABLE 1 Number and percentage of identified metabolite quantities that were significantly different in the *A. nidulans* clinical isolates[a]

	No. (%) of differentially produced metabolites	
Carbon source (*n*)[b]	MO80069 vs FGSC A4	SP-2605-48 vs FGSC A4
Glucose (40)	18 (45)	15 (38)
Ethanol (40)	22 (55)	23 (58)
Acetate (44)	23 (52)	30 (68)
Mucin (44)	24 (55)	14 (32)

[a]Metabolite quantities in *A. nidulans* clinical isolates were compared to those of the reference strain ($P < 0.05$). Strains were grown in the presence of the indicated carbon source for 16 h.
[b]*n*, number of metabolites tested.

Metabolomics was carried out on cellular extracts from strains grown for 24 h in fructose-rich MM and then transferred for 16 h to MM supplemented with glucose (CIs present reduced growth), ethanol (CIs had increased growth), acetate, and mucin (no difference in growth profiles). A total of 40 different metabolites were identified when strains were grown in the presence of glucose and ethanol, whereas 44 different metabolites were identified when strains were grown in the presence of acetate and mucin (see Table S1; all supplemental material is posted at https://doi.org/10.6084/m9.figshare.11973936). In a comparison of the metabolite quantities of strain MO80069 to those of the reference strain, 18 (45%), 22 (55%), 23 (52%), and 24 (55%) metabolite quantities were significantly (*P* value of <0.05) different from the quantities in the reference strain when strains were grown in glucose, ethanol, acetate, and mucin, respectively (Table 1; see also Table S1). In strain SP-2505-48, 15 (38%), 23 (58%), 30 (68%), and 14 (32%) metabolite quantities, which were normalized by fungal dry weight, were significantly (*P* value of <0.05) different from the quantities in the reference strain in the presence of glucose, ethanol, acetate, and mucin, respectively (Table 1; see also Table S1). Principal-component analysis (PCA) and hierarchical clustering analysis (HCA) of identified metabolite quantities showed that the CIs clustered apart from the reference strain and from each other in all tested carbon sources (see Fig. S1 and S2 at the URL mentioned above), indicating that they are metabolically different from the reference strain and from each other.

When we further focused on metabolites that were significantly different in quantity between the CIs and the reference strain, we observed that in the presence of glucose and ethanol, the majority of identified metabolites were present in significantly lower quantities than in the reference strain whereas both CIs had significantly higher metabolite quantities in the presence of acetate than the reference strain (Fig. 2A to C). Furthermore, when the *A. nidulans* CIs were cultivated in mucin-rich minimal medium, only 9 out of 29 significantly different metabolite quantities were identified in both strains whereas the remaining metabolite quantities were strain specific, suggesting that the metabolic profiles of the two differed drastically in the presence of this carbon source (Fig. 2D).

When the CIs were grown in a glucose-rich MM, amino acids were found in lower quantities in both CIs than in the reference strain. In contrast, pentose phosphate pathway (PPP) intermediates, glycerol, glycerol derivatives, and aromatic amino acids were detected in significantly higher quantities in this carbon source (Fig. 2A). In an ethanol-rich MM, significantly lower quantities of various amino acids as well as of the citric acid cycle intermediate citrate were detected in the CIs whereas increased quantities of several amino acid pathway intermediates, the carbon compounds glycerol, mannitol, and trehalose, PPP intermediates, and lactate were detected in the CIs compared to levels in the reference strain grown in this carbon source (Fig. 2C). In acetate-rich MM, most identified metabolites, notably a variety of amino acids, were present in significantly larger amounts in the CIs than in the reference strain, with the exception of some amino acids, PPP intermediates, spermidine, rhamnose, and urea (Fig. 2B). When strains were grown in mucin-rich MM, differences in the quantities of a variety of amino acids were observed, whereas trehalose was present in significantly

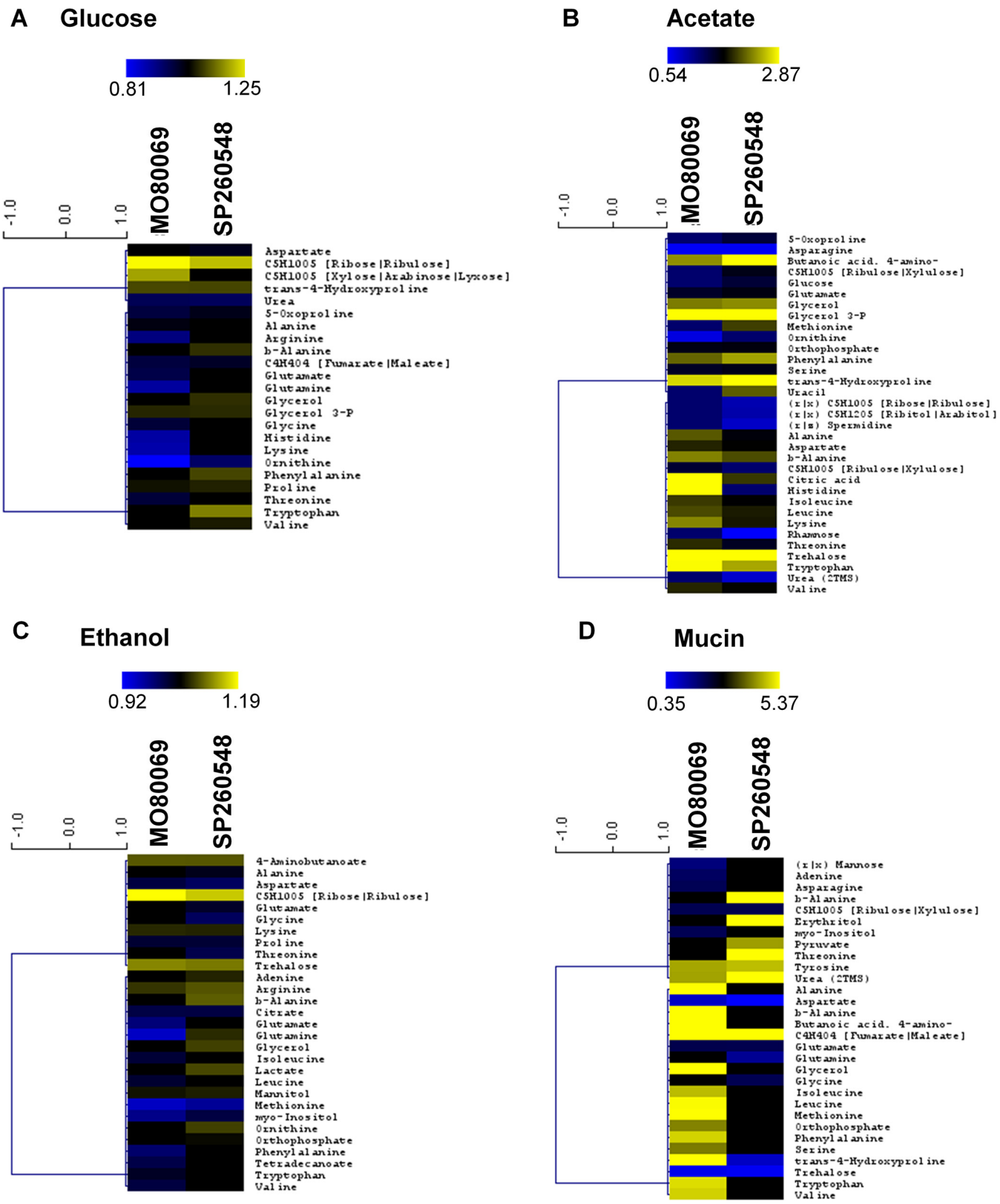

FIG 2 The *A. nidulans* clinical isolates are metabolically different from the reference strain in the presence of different carbon sources. (A to D) Heat maps depicting log fold changes of identified metabolite quantities that were significantly ($P < 0.05$) different in the *A. nidulans* clinical isolates MO80069 and SP-2605-48 compared to levels in the FGSC A4 reference strain (gray squares depict metabolite quantities that were not detected as significantly different in one of the clinical isolates).

TABLE 2 Significant metabolic pathway enrichments

	Enriched pathways in:	
Carbon source	**MO80069**	**SP-2605-48**
Glucose	Aminoacyl-tRNA biosynthesis, arginine and proline metabolism	Aminoacyl-tRNA biosynthesis, arginine and proline metabolism
Acetate	Aminoacyl-tRNA biosynthesis; alanine, aspartate, and glutamate metabolism; cyanoamino acid metabolism; valine, leucine, and isoleucine metabolism; glycine, serine and threonine metabolism	Aminoacyl-tRNA biosynthesis, beta-alanine metabolism
Ethanol	Aminoacyl-tRNA biosynthesis, arginine and proline metabolism	Aminoacyl-tRNA biosynthesis, arginine and proline metabolism, nitrogen metabolism, alanine, aspartate, and glutamate metabolism

lower quantities and urea was present in significantly higher quantities in both CIs than in the reference strain (Fig. 2D). In summary, these results suggest significant differences in amino acid biosynthesis and degradation, carbon source storage compounds, and degradation among the different *A. nidulans* strains in a condition-dependent manner.

To determine if any metabolic pathways were specifically enriched in the *A. nidulans* CIs in comparison to levels in the reference strain, pathway enrichment analyses were carried out on the metabolome data from glucose-, ethanol-, acetate-, and mucin-grown cultures. In all tested carbon sources, with the exception of mucin for isolate SP-2605-48, there was significant enrichment for aminoacyl-tRNA biosynthesis (Table 2). The pathway constituting the metabolism of arginine and proline was significantly enriched in both clinical isolates when they were grown in the presence of glucose and ethanol and in isolate SP-2605-48 when it was incubated in mucin-rich medium (Table 2). When acetate was used as the sole carbon and energy source, enrichment of the metabolism of these amino acids was not observed (Table 2). In addition, metabolites identified for strain SP-2605-48 in the presence of mucin and ethanol showed pathway enrichment in nitrogen metabolism (Table 2). In agreement with the aforementioned differences in amino acid quantities, these results suggest that the CIs exhibit differences in nitrogen metabolism in a carbon source-independent manner compared to that of the reference strain.

The *A. nidulans* clinical isolates are more sensitive to hydrogen peroxide-induced oxidative stress and cell wall-perturbing agents than the reference strain. Due to the significant metabolic differences observed between the CIs and the reference strain in the presence of physiologically relevant carbon sources and given that primary metabolism (carbon source utilization) has been shown to impact virulence factors in opportunistic pathogenic fungi (28, 29), we hypothesized that similar differences could be observed in the presence of physiologically relevant stress conditions. One such virulence factor is the fungal cell wall, which is crucial for protection, interaction with, and modulation or evasion of the host immune system (30). In addition, cell wall polysaccharide composition is dependent on carbon source primary metabolism (28, 29, 31).

The production of reactive oxygen species (ROS), such as H_2O_2, and subsequent augmentation of cellular oxidative stress are strategies employed by the mammalian immune system to combat potential invading pathogenic microorganisms (14). The *A. nidulans* reference strain and the two CIs were therefore grown in the presence of hydrogen peroxide (H_2O_2) and the oxidative stress-inducing compound menadione. Both CIs were more sensitive (reduced growth) to high concentrations of H_2O_2 (see Fig. S3A at https://doi.org/10.6084/m9.figshare.11973936) whereas they were more resistant to menadione than the reference strain (see Fig. S3B). Furthermore, iron sequestration and elevated body temperature are additional physiological stress responses exerted by the host to prevent and/or control infection progression (32). Strains were

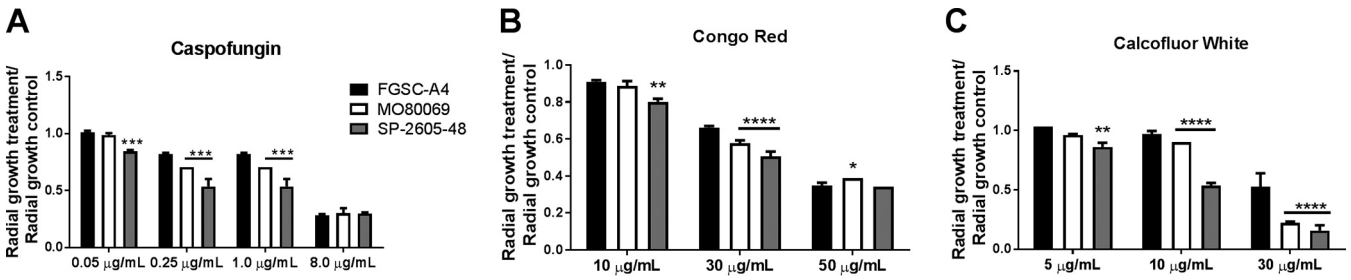

FIG 3 The *A. nidulans* clinical isolates are more sensitive to the cell wall-perturbing agents. (A to C) Strains were grown from 10^5 spores on glucose minimal medium supplemented with increasing concentrations of caspofungin, Congo red, and calcofluor white for 5 days at 37°C. Standard deviations represent biological triplicates in a two-way ANOVA test, comparing growth of the clinical isolates to growth of the FGSC A4 reference strain (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

therefore grown on iron-poor, glucose-rich minimal medium supplemented without (control) or with the iron chelators bathophenanthroline disulfonate (BPS) and ferrozine (see Fig. S3C), as well as in the presence of increasing temperatures (see Fig. S3D). Growth rates of all strains were similar under these conditions although strain MO80069 grew slightly more in the presence of the iron chelators (see Fig. S3C). Last, growth of all strains was assessed in the presence of the cell wall-perturbing agents caspofungin, Congo red (CR), and calcofluor white (CFW). The echinocandin caspofungin is a noncompetitive inhibitor of the cell wall enzyme β-1,3-glucan synthase (33) while CR and CFW bind to glucan and chitin chains, respectively (34, 35). CR and CFW therefore interfere with the cross-linking of cell wall polysaccharides, resulting in a reduction of cell wall stability. Both clinical isolates were more sensitive to low and medium concentrations of caspofungin than the reference strain, whereas all three strains grew similarly in the highest tested caspofungin concentration (8 μg/ml) (Fig. 3A). Similarly, both clinical strains were more sensitive to lower concentrations of CR whereas no significant difference in growth levels was observed in the presence of 50 μg/ml CR between all strains (Fig. 3B). In contrast, the CIs had significantly reduced growth in the presence of CFW compared to that of the reference strain (Fig. 3C).

In summary, the aforementioned results suggest strain-specific differences in the response to different physiological stress conditions and imply that the two *A. nidulans* CIs are more sensitive to cell wall-perturbing agents than the reference strain.

The *A. nidulans* clinical isolates do not display increased resistance to azoles and amphotericin B. Since both CIs showed increased susceptibility to caspofungin, an echinocandin that is being used as a second-line treatment for fungal infections (33), and to other cell wall-perturbing agents, we expanded our analyses to include two additional antifungal drugs classes. Specifically, we followed guidelines for the diagnosis and management of aspergillosis, which, in most cases, recommends treating aspergillosis with azoles and polyene drugs (11), both of which are known to interfere with the biosynthesis or physicochemical properties of fungal membrane sterols (10). Therefore, we determined the MICs of the azoles voriconazole and posaconazole and the polyene amphotericin B for all three strains. No differences in the MICs among all strains to these drugs was observed (Table 3).

Cleistothecium formation is impaired in the *A. nidulans* SP-2605-48 strain. *A. nidulans* is known for its easily inducible sexual cycle, which serves as a laboratory-

TABLE 3 MICs of voriconazole, posaconazole, and amphotericin B for the *A. nidulans* clinical isolates MO80069 and SP-2605-48 and the FGSC A4 reference strain

	MIC (μg/ml)		
Strain	Voriconazole	Posaconazole	Amphotericin B
FGSC A4	0.25	1.0	2.0
MO80069	0.25	1.0	2.0
SP260548	0.25	1.0	2.0

TABLE 4 Cleistothecium formation and density and ascospore viability resulting from diverse *A. nidulans* self- and outcrosses

Temp (°C)	Cross[a]	Cleistothecium production	Cleistothecium density (no. of cleistothecia/cm2)	Ascospore viability (%)
30	A4 × A4	Yes	15.0 ± 0.81	91.83 ± 3.53
	MO × MO	Yes	7.0 ± 1.35	92.83 ± 3.96
	SP × SP	Yes	0.25 ± 0.25	89.83 ± 3.51
	MO × R21	Yes	1.25 ± 0.25	94.83 ± 3.85
	SP × R21	No		
37	A4 × A4	Yes	9.75 ± 1.43	90.67 ± 3.62
	MO × MO	Yes	5.25 ± 1.31	92.5 ± 2.76
	SP × SP	No		
	MO × R21	Yes	5.0 ± 0.40	92.5 ± 1.28
	SP × R21	No		

[a]A4, FGSC A4 reference strain; MO, MO80069 clinical isolate; SP, SP-2605-48 clinical isolate; R21, R21XR135, *paba*-deficient strain.

based molecular tool for strain construction and studying fungal sexual reproduction (36). To further characterize *A. nidulans* CI biology, we performed self- and outcrosses for each clinical strain and the reference strain (control) at 30 and 37°C to assess whether *A. nidulans* CIs are able to undergo sexual reproduction.

Strains were first crossed with themselves (self-crosses) at 30°C and 37°C, and cleistothecium formation was observed for all strains at both temperatures, except for strain SP-2605-48 at 37°C (Table 4). Density of cleistothecia (number of cleistothecia/square centimeter) also varied between strains in a temperature-dependent manner, with the clinical isolates forming fewer cleistothecia per square centimeter than the reference strain at 30°C and 37°C (Table 4). In addition, no difference in levels of ascospore viability was observed among strains (Table 4).

Outcrosses were performed by crossing the *pyrG* (requirement for uridine and uracil) auxotrophic strains MO80069 and SP-2605-48 with the *paba* (requirement for para-aminobenzoic acid)-deficient strain R21XR135 (Table 5). Strain MO80069 produced cleistothecia at both 30 and 37°C whereas strain SP-2605-48 did not produce any cleistothecia under any of the tested conditions. Density of cleistothecia was very low at 30°C (1.25 cleistothecia/cm^2) but increased to the same number observed for the self-crosses at 37°C, with high ascospore viability in all cases (Table 4).

Identification of SNPs and copy number variations in the *A. nidulans* clinical isolate genomes. The aforementioned phenotyping and metabolomics results indicate differences between the strains that affect traits such as nutrient source utilization and resistance to different stresses. These results are in agreement with studies in *A. fumigatus* that have described great strain heterogeneity in traits such as growth, fitness, and enzyme secretion between different environmental and clinical isolates (24, 37). Indeed, the number of single nucleotide polymorphisms (SNPs), obtained during strain pairwise comparison, in the genomes of different *A. fumigatus* strains range

TABLE 5 Strains used in this study

Strain	Genotype	Source	Reference
FGSC-A4	Glasgow wild type (*veA*$^+$)	Soil	36
MO80069	Wild type, clinical isolate	Bronchoalveolar lavage sample from a patient with breast carcinoma and pneumonia (Portugal)	This study
SP-2605-48	Wild type, clinical isolate	Patient with cystic fibrosis who underwent lung transplantation (Belgium)	This study
R21XR135	*pabaA1 yA2*	NA[a]	This study
MO80069 *pyrG*$^-$	*pyrG89*	This study	This study
SP-2605-48 *pyrG*$^-$	*pyrG89*	This study	This study
Δ*mpkA* strain	Δ*akuB mpkA*::*ptrA PTR*	NA	75

[a]NA, not applicable.

TABLE 6 Type and number of SNPs and long indels detected between the genomes of the *A. nidulans* clinical isolates MO80069 and SP-2605-48 and compared to the FGSC A4 reference genome

Mutation type	No. of mutations		
	MO80069 vs FGSC A4	SP-2605-48 vs FGSC A4	SP-2605-48 vs MO80069
SNPs			
Stop codon gain/loss	149	110	170
Frameshift	352	355	256
Missense	6,271	5,896	6,288
Synonymous	6,184	6,038	6,122
Total	12,956	12,399	12,836
Indels			
Insertions	234	308	222
Deletion	114	138	207
Total	348	446	375

between ~13,500 (24) and ~50,000 (38, 39). Strain heterogeneity has therefore mainly been investigated in environmental and clinical isolates of *A. fumigatus*, whereas similar studies have not been carried out for *A. nidulans* isolates. We therefore decided to determine differences at the genomic level by sequencing the genomes of our two *A. nidulans* CIs and comparing the sequences to the sequence of the FGSC A4 reference genome.

The genomes of MO80069 and SP-2605-48 aligned at 98.3% and 97.4%, respectively, to the genome of the reference strain FGSC A4, with 99.8% nucleotide identity. On the other hand, 1.5% and 1.9% of the A4 assembled genome did not align to the MO80069 and SP-2605-48 genomes, respectively, indicating differences among the genomes of all three strains.

A total of 12,956 and 12,399 SNPs with respect to the A4 reference genome were detected in the genomes of MO80069 and SP-2605-48, respectively (Table 6; see also Table S2 at https://doi.org/10.6084/m9.figshare.11973936). When the genome of SP-260548 was compared to the genome of MO80069, 12,836 SNPs were detected (Table 6; see also Table S2). Each SNP mutation was classified as either high, moderate, or low according to its impact on the DNA codon frame and amino acid sequence. High-impact-type mutations encompass frameshift mutations and stop codon gain/loss, whereas missense mutations, resulting in amino acid changes, are considered moderate-impact-type mutations. Low-impact-type mutations contain all synonymous mutations and mutations within gene introns and untranslated regions (UTRs). The genome of MO80069 contained 501 high-impact mutations, 6,271 missense (moderate impact) mutations, and 6,184 synonymous (low impact) mutations in comparison to the sequence of the reference genome (Table 6; see also Table S2). In the genome of SP-2605-48, 465 high-impact mutations, 5,896 moderate-impact mutations, and 6,038 low-impact mutations were detected in comparison to the sequence of the reference genome (Table 6; see Table S2). When the genomes of both CIs were compared, 426 high-impact mutations, 6,288 missense mutations, and 6,122 synonymous mutations were detected (Table 6; see also Table S2 at the URL mentioned above). All nonsynonymous mutations were distributed throughout the genomes of both CIs, and no clear pattern in mutation accumulation could be observed for any of the 8 chromosomes (Fig. 4 and 5).

In addition, the genomes of both CIs were screened for large-scale (>50 bp) insertions and deletions (indels). In total, 1,169 large-scale indels, consisting of anything between 3 bp to 23 kbp in size, were detected among the eight chromosomes of the CIs compared to the genome of the reference strain (see Table S3). Of these, 348 indels were specifically located in the genome of MO80069, 446 indels were found in the genome of SP-2605-48 only, and 375 indels were located in the genomes of both CIs (Table 6; see also Table S3). The majority of these indels were insertions (Table 6). Of the 375 indels found in the genomes of both CIs, 227 (60.5%) indels differed between the

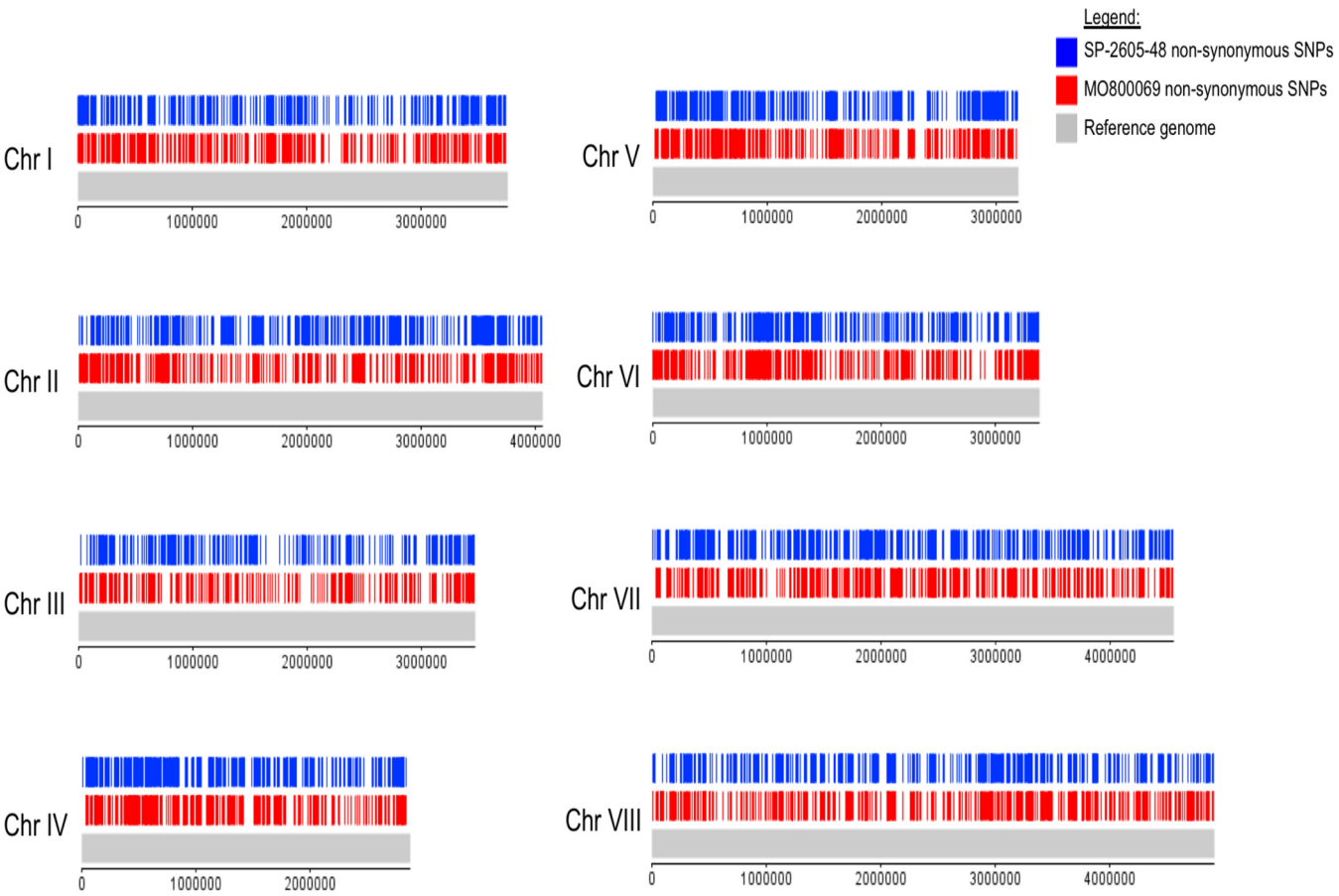

FIG 4 Diagram depicting the location of all detected nonsynonymous single nucleotide polymorphisms (SNPs) on the 8 chromosomes (Chr I to Chr VIII) of the *A. nidulans* clinical isolates SP-2605-48 and MO80069 in comparison to the FGSC A4 reference genome.

two strains, with the remaining 148 indels being identical for both strains (see Table S3 at the URL mentioned above).

The *A. nidulans* clinical isolates are defective in MpkA accumulation in response to cell wall stress. As this work aimed to characterize metabolic utilization of physiologically relevant carbon and lipid sources in *A. nidulans* CIs, including acetate and fatty acids, we screened genes encoding proteins important for carbohydrate and lipid utilization, cell wall biosynthesis/remodeling, and sexual reproduction for the presence of any of the aforementioned moderate- and high-impact mutations (see Table S4 at the URL mentioned above). Moderate-impact (missense) mutations were detected in three genes (*hxkA*, *swoM*, and *pfka*), encoding proteins involved in glycolysis (hexokinase, glucose-6-phosphate isomerase, and 6-phosphofructokinase) in both CIs, whereas four and six missense mutations were found in two genes (*idpA* and *mdhA*) encoding the enzymes isocitrate dehydrogenase and malate dehydrogenase of the tricarboxylic acid cycle in the genomes of MO80069 and SP-2605-48, respectively (see Table S4). Similarly, several moderate-impact mutations were found in genes encoding enzymes required for C_2-associated metabolism (acetate, ethanol, and fatty acid), including *farA* (transcription factor regulating fatty acid utilization) and *farB* (transcription factor regulating the utilization of short-chain fatty acids) in both CIs, *facA* (acetyl-coenzyme A [CoA] synthase), *acuM* (transcriptional activator required for gluconeogenesis), and *alcM* (required for ethanol utilization) in SP-2605-48, and *echA* (enoyl-CoA hydratase) in MO80069 (see Table S4). Genes encoding proteins that function in the glyoxylate cycle also contained missense mutations in both CIs (see Table S4). Furthermore, a frameshift mutation was detected in both CIs in *acuL*, encoding a mitochondrial carrier involved in the utilization of carbon sources that are

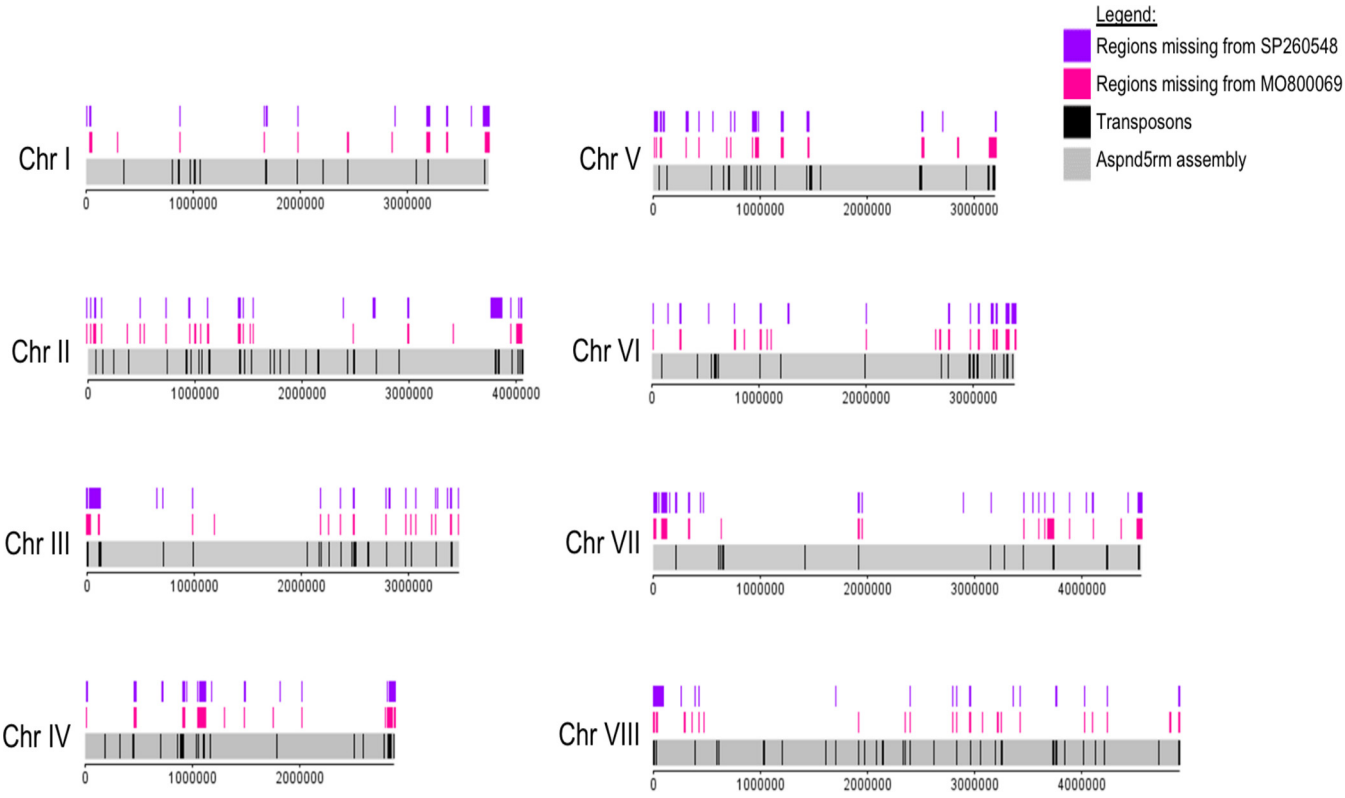

FIG 5 Diagram depicting the location of all detected small deletions on the 8 chromosomes (Chr I to Chr VIII) of the *A. nidulans* clinical isolates SP-2605-48 and MO80069 in comparison to the FGSC A4 reference genome. Also shown are the locations of putative transposons in the *A. nidulans* reference genome.

metabolized via the Krebs cycle (40) (see Table S4 at the URL mentioned above). The aforementioned mutations could underlie the observed differences in phenotypic growth in the presence of different carbon and lipid sources.

Due to the absence of cleistothecium formation in strain SP-2605-48, we wondered whether this strain contained any mutations in genes encoding proteins required for *A. nidulans* sexual reproduction. We found 11 and 13 mutations in 7 and 9 genes related to mating in the MO80069 and SP-2605-48 genomes, respectively (see Table S4). These mutations include missense and frameshift mutations in genes involved in the perception of light and dark (*ireA*, *ireB*, *cryA*, *veA*, and *velB*), mating processes (*cpcA*, *rosA*, and *nosA*), and signal transduction (*gprH* and *gprD*) (see Table S4). Indeed, *rosA* was absent in both CIs whereas *ireA* was missing from the genome of SP-2605-48. RosA is a transcriptional repressor of sexual development (41) whereas IreA is a transcription factor required for the blue light response, important for developmental processes, including mating.

Last, as both CIs were sensitive to cell wall-perturbing agents, we screened for mutations in genes encoding enzymes involved in cell wall biosynthesis and degradation. Compared to the FGSC A4 reference genome, we found 159 and 90 mutations in 40 and 34 genes involved in cell wall biosynthesis, integrity, and signaling in the genomes of MO80069 and SP-2605-48, respectively (see Table S4 at https://doi.org/10.6084/m9.figshare.11973936). The majority of these mutations were moderate-impact missense mutations in genes that encode components required for 1,3-β- and α-glucan and chitin synthesis and degradation, including various types of glucanases, chitinases, and chitin synthases (see Table S4). However, 17 (MO80069) and 9 (SP-2605-48) mutations were high-impact-level mutations which occurred in genes AN0550 (putative glucan 1,3-beta-glucosidase), AN0509 (putative chitinase), AN0517 (putative chitinase), AN0549 (putative chitinase), AN9042 (putative alpha-1,3-glucanase), AN6324 (putative α-amylase), AN4504 (putative endo-mannanase), and AN0383 (putative endo-mannanase)

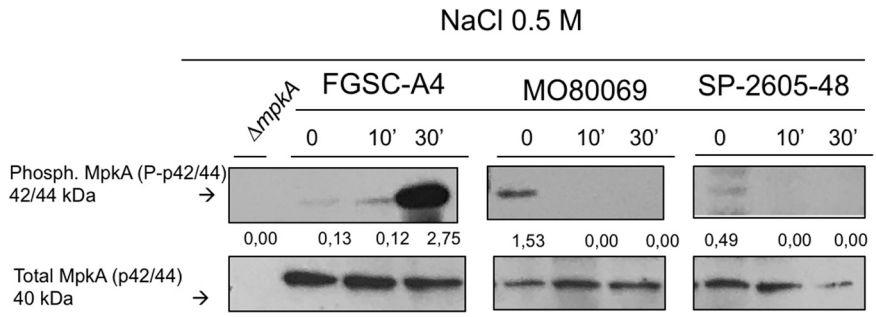

FIG 6 MpkA is not phosphorylated in the *A. nidulans* clinical isolates MO80069 and SP-2605-48 in the presence of NaCl-induced cell wall stress in contrast to MpkA levels in the FGSC A4 reference strain. Strains were grown from 1×10^7 spores in complete medium for 16 h (control, 0 min) at 37°C before 0.5 M NaCl was added for 10 min (10′) and 30 min (30′). Total cellular protein was extracted, and Western blotting was carried out probing for phosphorylated MpkA. Signals were normalized by the amount of total MpkA present in the protein extracts, and cellular extracts from the Δ*mpkA* strain were used as a negative control.

(see Table S4). In addition, small frameshift mutations were detected in three genes encoding the mitogen-activated protein kinase (MAPK) kinase BckA (AN4887), the MAPK MpkA (AN5666), and the transcription factor RlmA (AN2984) (see Table S4). In *A. fumigatus*, BckA and MpkA are components of the cell wall integrity (CWI) pathway, which ensures the integrity of the cell wall and is activated in response to different cell wall stresses, including those exerted by cell wall-targeting antifungal drugs (42). RlmA was shown to act downstream of MpkA, regulating cell wall biosynthesis-related genes, and this transcription factor is also involved in the direct regulation of MpkA (43). Mutation in *rlmA* was observed only in the genome of strain SP-2648-05.

In order to determine whether the observed frameshift mutations had an impact on CWI signaling, we carried out Western blotting of phosphorylated MpkA in the presence of NaCl-induced cell wall stress in all three *A. nidulans* strains. Phosphorylated MpkA levels were normalized by total cellular MpkA. Low levels of phosphorylated MpkA were detected in the absence of NaCl in all three strains, but whereas MpkA protein levels significantly increased upon cell wall stress in the FGSC A4 reference strain, no phosphorylated MpkA could be detected in either CI (Fig. 6). These results suggest that the observed frameshift mutations in *mpkA* had an effect on MpkA protein levels in the presence of cell wall stress, potentially being (one of) the cause(s) for the observed increased sensitivity to cell wall-perturbing agents.

The *A. nidulans* clinical isolates do not display increased resistance to *in vitro*-mediated killing by different types of macrophages and neutrophils. Due to the observed phenotypic and genotypic differences, we wondered whether the CIs were different in virulence from the reference strain. Virulence was first characterized under a variety of *in vitro* conditions. Macrophages play an essential role in clearing *Aspergillus* species conidia from the lung (8), whereas neutrophils are predicted to primarily be responsible for eliminating fungal hyphae (39). To determine whether any strain-specific differences exist in macrophage-mediated phagocytosis and killing, the respective assays were carried out for all three strains in the presence of murine wild-type and gp91^{phox} knockout (CGD) macrophages. Macrophages from CGD patients are impaired in eliminating conidia from the lung environment, thus rendering the host more susceptible to fungal infections (20). Both types of macrophages phagocytosed a significantly higher number of conidia from both *A. nidulans* clinical isolates (~75%) than the reference strain (~50%) (Fig. 7A). Indeed, conidia from all three *A. nidulans* strains had increased levels of viability after phagocytosis by gp91^{phox} knockout macrophages than wild-type macrophages, confirming the inability of this type of macrophage to efficiently kill fungal conidia (Fig. 7B). Despite increased phagocytosis of both CIs, no difference in conidial viability levels was observed for strain MO80069 compared to the level of the reference strain, whereas wild-type but

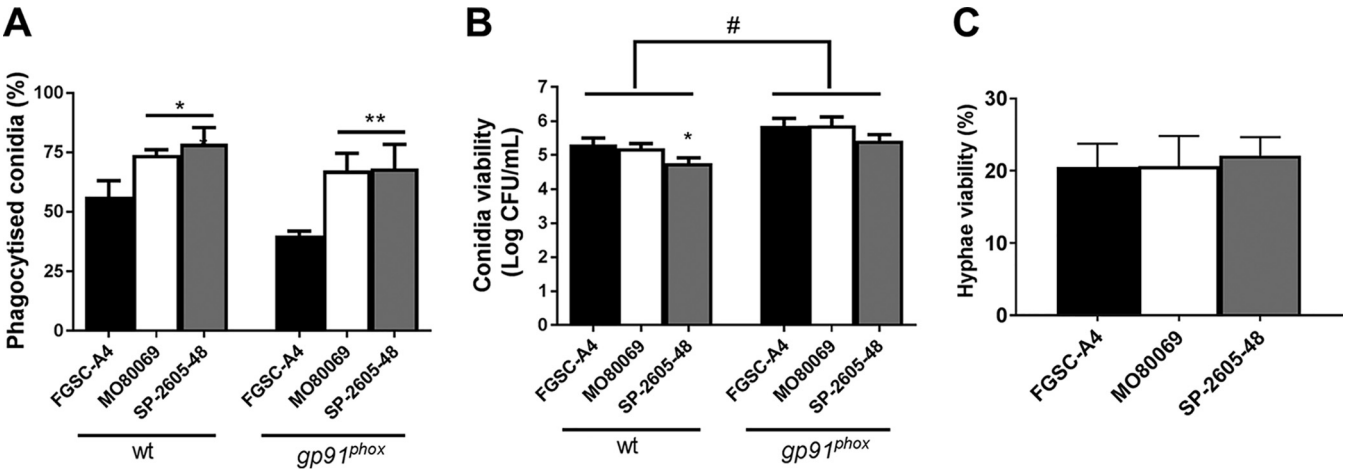

FIG 7 The *A. nidulans* clinical isolates MO80069 and SP-2605-48 do not present increased survival in the presence of macrophages and neutrophils. (A) Percentage of phagocytized conidia by murine wild-type and gp91^{phox} knockout macrophages. Macrophages were incubated for 1.5 h with conidia from the respective strains before phagocytized conidia were counted. (B) CFU counts as a measure of conidium viability after passage through wild-type (wt) and gp91^{phox} knockout macrophages. Macrophages were incubated with the respective conidia for 1.5 h before they were lysed, and contents were plated on complete medium. (C) Percentage of viable hyphal germlings after incubation for 16 h with neutrophils from healthy human donors. Strain viability was calculated relative to incubation without PMN cells, which was set at 100% for each sample. Standard deviations represent biological triplicates in a one-way ANOVA test with Tukey's posttest (*, $P < 0.05$; **, $P < 0.01$, for results for the clinical isolates compared to those for FGSC A4; #, $P < 0.05$, for a comparison of results for the two types of macrophages in the same strain).

not CGD macrophages succeeded in killing significantly more SP-2605-48 conidia (Fig. 7B).

When challenged with human polymorphonuclear (PMN) cells, fungal survival was reduced approximately 80% for all three *A. nidulans* strains, indicating that the neutrophils were actively killing the hyphal germlings (Fig. 7C). No difference in strain survival rates was observed for the CIs (Fig. 7C). These results suggest that the *A. nidulans* CIs do not have higher survival rates in the presence of macrophages and neutrophils.

Virulence of the *A. nidulans* clinical isolates depends on the host immune status. We determined the virulence of both *A. nidulans* CIs in animal models with different immune statuses. As it is well known that *A. fumigatus* strain-specific virulence is highly dependent on the type of host immunosuppression and model (24, 37, 43), we sought to determine if this would also be the case for *A. nidulans*. The virulence of *A. nidulans* CIs was assessed in both zebrafish and murine models of pulmonary and invasive aspergillosis. Furthermore, the immune system of each animal was manipulated in order to give rise to either immunocompetent, CGD, or neutropenic/neutrophilic models. As with patients, CGD models of both mice (19) and zebrafish (21) are very susceptible to *A. nidulans* infections. In both immunocompetent- and CGD-type zebrafish and mice, no difference in virulence levels between the *A. nidulans* clinical isolates and the reference strain was observed (Fig. 8A to D). However, the CI MO80069 was significantly more virulent in neutropenic mice and zebrafish with impaired neutrophil function than the reference strain, whereas no difference in virulence was observed for strain SP-2605-48 (Fig. 8E and F). These results suggest that, as in *A. fumigatus*, *A. nidulans* virulence depends on the strain and the host immune status.

DISCUSSION

Aspergillus nidulans is a saprophytic fungus that can act as an opportunistic human pathogen in a host immune status- and genetic condition-dependent manner (15, 18, 44). Infection with *A. nidulans* is prevalent in patients with chronic granulomatous disease (CGD), and isolates have mainly been characterized in the context of this disorder (14, 15). Studies on *A. nidulans* virulence have been carried out in CGD models (animal and cell culture), and virulence characteristics have been compared to those of the primary human opportunistic fungus *A. fumigatus* (20, 21, 45, 46). *A. fumigatus*

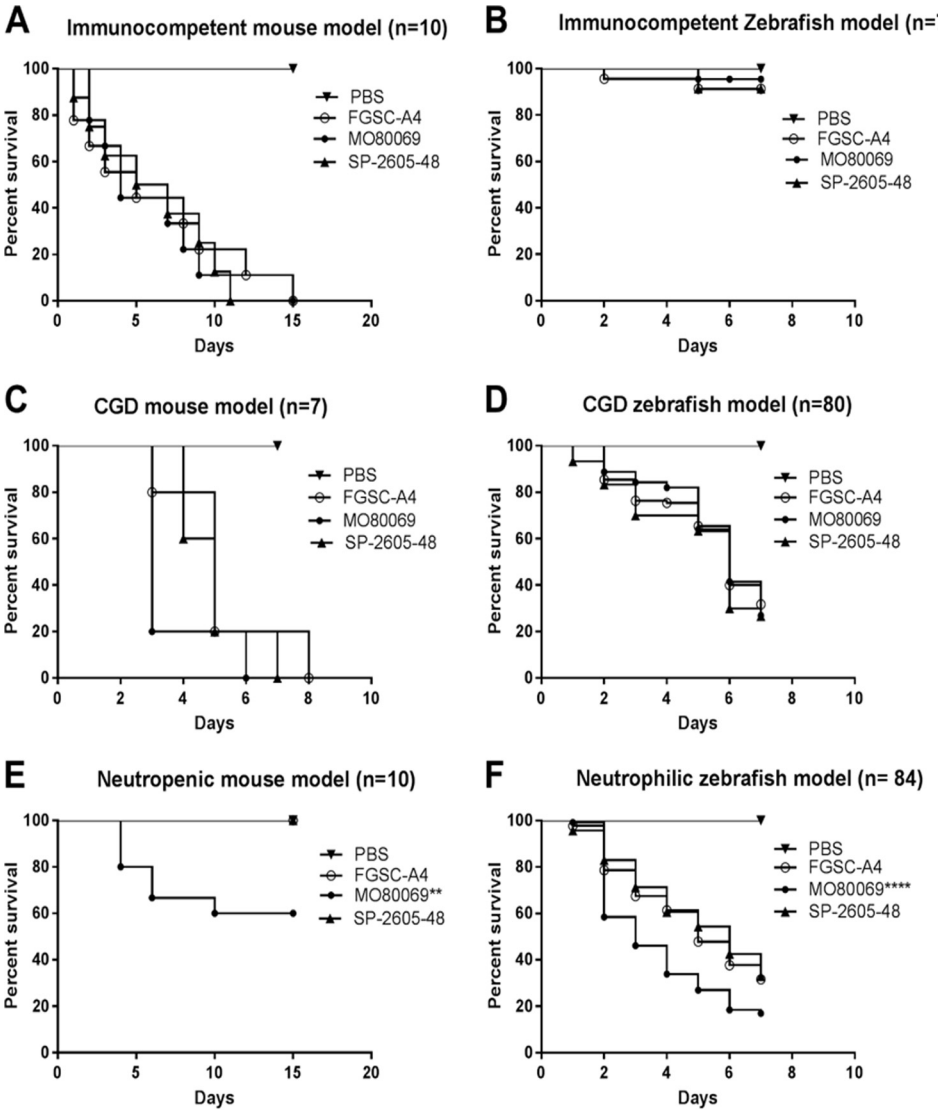

FIG 8 *A. nidulans* strain-specific virulence depends on the host immune status. The virulence of the *A. nidulans* clinical isolates MO80069 and SP-260548 was tested in murine (A, C, and E) and zebrafish (B, D, and F) models of pulmonary and invasive aspergillosis. Animals were manipulated in order to give rise to either immunocompetent (A and B), CGD (chronic granulomatous disease) (C and D), or neutropenic (E)/neutrophilic (F) models. Shown are survival curves for each immunosuppression condition and animal model. No difference in virulence levels was detected for all strains in both immunocompetent and CGD mice. Strain MO80069 was significantly more virulent in neutropenic mice and neutrophilic zebrafish. **, $P < 0.01$; ****, $P < 0.0001$ for a comparison of the values for the clinical isolates to those of the FGSC A4 reference strain in a two-way ANOVA test with Tukey's posttest.

infection biology and characterization of strains that were isolated from immunocompromised patients under different conditions have received considerable attention in recent years (24, 37, 47), whereas similar studies into other pathogenic *Aspergillus* spp. have been neglected although it is becoming apparent that non-*A. fumigatus* species, including cryptic *Aspergillus* species, also contribute to host infection and invasion (7). This work therefore aimed at providing a detailed phenotypic, metabolic, genomic, and virulence characterization of two *A. nidulans* clinical isolates (CIs) that were isolated from non-CGD patients.

The first CI (MO80069) was isolated from a patient with breast carcinoma and pneumonia whereas the second CI (SP-2605-48) was obtained from a patient with cystic fibrosis who underwent lung transplantation. Genome sequencing confirmed these strains to be *A. nidulans sensu stricto*, and growth of these strains was characterized in

the presence of physiologically relevant carbon sources. Fungi require carbon sources in large quantities in order to sustain biosynthetic processes and actively scavenge for them in their environment, including mammalian hosts (24). Available carbon sources vary according to the patient's immune status and disease progression with, for example, corticosteroid treatment resulting in an increase of fatty and amino acid concentrations and a decrease of glucose levels in mouse lungs (22). Growth of the two *A. nidulans* strains in the presence of different carbon sources differed significantly from growth of the reference strain, with increased biomass accumulation being observed in the presence of alternative (ethanol, lipids, and amino acids) carbon sources and reduced growth in the presence of glucose. The observed phenotypic differences were corroborated by metabolic and genomic data which found a number of missense and high-impact mutations in genes encoding enzymes required for alternative carbon source and glucose utilization. These included missense mutations in genes encoding glycolysis- and citric acid cycle-related enzymes as well as five missense mutations in the transcription factor-encoding gene *farA*, which regulates the utilization of short- and long-chain fatty acids. Whether these mutations alone and/or in combination with other identified gene mutations are responsible for the observed growth phenotypes remains to be determined. Nevertheless, it is noteworthy that these mutations are found in both CIs, suggesting that these strains are able to grow well in nutrient-poor environments, such as the lung, compared to growth of the reference strain, which was isolated from the soil environment. Furthermore, whether these mutations are a result of adaptation to the host environment also remains subject to future investigations.

In addition, we also assessed the resistance of these strains to a variety of physiologically relevant stress conditions by growing them in the presence of oxidative stress and cell wall stress-inducing compounds, high temperature, iron limitation, and antifungal drugs. Some minor strain-specific differences were observed under these conditions, but the CIs were not significantly more resistant to these conditions, including exposure to azole- and polyene-type anti-fungal drugs, than the reference strain. It is possible that the patient-specific lung environment, biofilm formation, and/or interactions with other microorganisms may result in protection of these stresses, thus resulting in strains that do not have increased stress tolerance. In contrast to *Candida albicans*, an opportunistic fungal pathogen which was shown to interact with the Gram-negative bacterium *Pseudomonas aeruginosa* to promote colonization of patients with cystic fibrosis in a condition-dependent manner (48), such interactions have not been investigated for *Aspergillus* spp. *Aspergillus* interspecies interaction in lung microbiomes of patients with and without cystic fibrosis therefore remains an intriguing aspect of fungal pathobiology that warrants further characterization.

In contrast, both *A. nidulans* clinical strains were significantly more sensitive to the cell wall-perturbing agents calcofluor white, Congo red, and caspofungin (33–35) than the reference strain. These results suggest differences in cell wall composition and/or organization between the clinical isolates and the reference strain. When the respective genome sequences were analyzed, we found 159 and 90 mutations in 40 and 34 genes encoding enzymes required for cell wall glucan and chitin biosynthesis and degradation in strains MO80069 and SP-2605-48, respectively, compared to the genome of the FGSC A4 reference strain. Of particular interest was the identification of high-impact mutations in the genes *bckA*, *mpkA*, and *rlmA*, which encode components of the CWI signaling pathway. Indeed, Western blotting confirmed the absence of MpkA phosphorylation in the CIs in the presence of cell wall stress. These results suggest that the observed gene mutations cause an altered CWI response, resulting in increased sensitivity to cell wall-perturbing agents. The physiological relevance of these findings remains to be determined.

Aspergillus nidulans is characterized by an easily inducible sexual cycle as well as by undemanding laboratory-based cultivation and genetic manipulation conditions and has extensively been used as a model organism to study sexual reproduction and developmental processes (49). Nevertheless, it is unknown whether these traits can also be applied to *A. nidulans* clinical strains, and this work therefore assessed the ability of

the two CIs to form cleistothecia in self- and outcrosses. Strain MO80069, similar to the reference strain, produced cleistothecia and viable ascospores under all tested conditions, whereas strain SP-2605-48 formed cleistothecia and viable ascospores only in self-crosses at 30°C and not at 37°C. This suggests that a certain degree of heterogeneity exists with regard to sexual reproduction in *A. nidulans* clinical strains although a bigger sample size and further studies are required in order to confirm this. Temperature has been shown to influence cleistothecium formation in *Aspergillus* spp., with lower temperatures of 30°C resulting in a higher number of formed cleistothecia (50). Furthermore, we cannot exclude the possibility that strains such as SP-2605-48 may require a different condition for sexual reproduction as it is determined by a series of environmental factors that can either activate or repress sexual development (50). This work identified six missense mutations in four genes (*veA*, *cpcA*, *fhbB*, and *gprH*) encoding enzymes involved in sexual development, and gene *ireA* was absent in the SP-2605-48 genome compared to the genomes of strains FGSC-A4 and MO80069. Genes *veA*, *cpcA*, *fhbB*, and *ireA* encode proteins that are involved in the perception of environmental signals (50), favoring the hypothesis that SP-2605-48 may require different/specific conditions for cleistothecium production, although it remains to be determined whether the aforementioned mutations and *ireA* are directly linked to the absence of cleistothecium production in strain SP-2605-48 under the conditions tested here.

Last, this work examined the *in vivo* virulence of the *A. nidulans* CIs in different animal models with a variety of immune statuses as *A. fumigatus* strain-specific virulence is highly dependent on the type of host immunosuppression and model (24, 37, 51). No difference in virulence levels was observed in immunocompetent and CGD murine and zebrafish models whereas strain MO80069 was significantly more virulent in a zebrafish with impaired neutrophil function and a neutropenic murine model of invasive aspergillosis than strains FGSC A4 and SP-2605-48. These results suggest that neutrophil recruitment and function at the site of infection are important for controlling *A. nidulans* infection in both vertebrates. Furthermore, results are in agreement with studies on *A. fumigatus*, which show that virulence is as much a strain-dependent as a host-dependent trait (24, 37, 39, 51). Furthermore, the tested phenotypes and genome mutations appear not to correlate with strain virulence although sample size has to be increased in order to confirm this in future studies. *Aspergillus* infection biology of mammalian hosts is a multifactorial and multifaceted process that depends not only on strain-specific virulence traits (30) but also on the genetic composition of the host and status of the immune system (52). Furthermore, the composition and interspecies interactions of the lung microbiome also influence pathogenicity of a given microorganism, with interactions between different species shown to influence host immune responses (49, 53). *A. fumigatus* is the main etiological agent of *Aspergillus*-related diseases and is predominantly present in the lung environment compared to the sites of infections caused by *Aspergillus* spp. (7). It is therefore possible that other *Aspergillus* spp., such as *A. nidulans*, remain largely undetected in the lung environment due to the predominant nature and/or inhibitory function of other fungal species and where they can grow without the necessity to evolve and adapt to extreme stress conditions. The prevalence and virulence of non-*A. fumigatus* species therefore remains a highly interesting and somewhat neglected topic that warrants future detailed studies. In summary, this is the first study that presents extensive phenotypic, metabolic, genomic, and virulence characterization of two *A. nidulans* clinical isolates. Just as in *A. fumigatus*, strain heterogeneity exists in *A. nidulans* clinical strains that can define virulence traits. Further studies are required to fully characterize *A. nidulans* strain virulence traits and pathogenicity.

MATERIALS AND METHODS

Ethics statement. The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO on the 27 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 adult and larval zebrafish proce-

dures were in full compliance with NIH guidelines and approved by the University of Wisconsin—Madison Institutional Animal Care and Use Committee (no. M01570-0-02-13).

Strains, media, and growth conditions. All strains used in this study are listed in Table 5. *A. nidulans* strain FGSC A4 was used as a reference strain. In addition to culture macroscopic features and fungal microscopic morphology analysis, whole-genome sequencing and phylogenetic analysis confirmed that both clinical isolates are *A. nidulans* (see Fig. S4; all supplemental material is available at https://doi.org/10.6084/m9.figshare.11973936). For phylogenetic tree construction, we compared *CaM*, *BenA*, *RPB2*, and *ITS* ribosomal DNA (rDNA) sequences, identified using blastN implemented in BLAST+, version 2.8.1 (54), to sequences from other species in the *Aspergillus* section *Nidulantes* (55), using a maximum-likelihood tree constructed with MEGA, version 10.1.1 (56). All strains were maintained in 10% glycerol at −80°C.

Strains were grown in either complete medium (CM) or minimal medium as described previously (57). Iron-poor MM was devoid of all iron and supplemented with 200 μM concentrations of the iron chelators bathophenanthrolinedisulfonic acid (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid [BPS]) and 300 μM 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine). All growth was carried out at 37°C for the indicated amounts of time, except where otherwise stated (Fig. 1 and 3; see also Fig. S3). Reagents were obtained from Sigma-Aldrich (St. Louis, MO) except where otherwise stated. Radial growth was determined by inoculating plates with 10^5 spores of each strain and incubation for 5 days before colony diameter was measured. Where required, the oxidative stress-inducing compound menadione or the cell wall-perturbing compounds Congo red (CR), caspofungin, and calcofluor white (CFW) were added in increasing concentrations. All radial growth was expressed as ratios, dividing colony radial diameter (in centimeters) of growth under the stress condition by colony radial diameter under the control (no stress) condition. To determine fungal dry weight, strains were grown from 3×10^6 spores in 30 ml of liquid MM supplemented with 1% (wt/vol) glucose, acetate, mucin, or Casamino Acids or 1% (vol/vol) ethanol, Tween 20 and 80, or olive oil for 48 h (glucose) or 72 h (others) at 37°C and 150 rpm. All liquid and solid growth experiments were carried out in biological triplicates.

Growth in the presence of H_2O_2 was carried out as serial dilutions (10^5 to 10^2 spores) in liquid CM in 24-well plates for 48 h in the presence of different concentrations of H_2O_2.

Metabolite analysis. Metabolome analysis was performed as described previously (58). Briefly, metabolites were extracted from 5 mg of dry-frozen, mycelial powder of four biological replicates. The polar phase was dried, and the derivatized sample was analyzed on a Combi-PAL autosampler (Agilent Technologies GmbH, Waldbronn, Germany) coupled to an Agilent 7890 gas chromatograph coupled to a Leco Pegasus 2 time-of-flight mass spectrometer (LECO, St. Joseph, MI). Chromatograms were exported from the Leco ChromaTOF software, version 3.25, to the R software package (www.r-project.org). The Target Search R package was used for peak detection, retention time alignment, and library matching.

Metabolites were quantified by the peak intensity of a selective mass and normalized by dividing the value by the respective sample dry weight. Principal-component analysis was performed using the pcaMethods bioconductor package (59, 60). Pathway enrichment analysis was carried out using MetaboAnalyst (https://www.metaboanalyst.ca/faces/ModuleView.xhtml) (61).

Determination of MICs. MICs of amphotericin B, voriconazole, and posaconazole were determined by growing 10^4 spores/well in 96-well plates containing 200 μl/well of RPMI medium and increasing concentrations of the aforementioned compounds, according to the protocol elaborated by the Clinical and Laboratory Standards Institute (62).

Induction of cleistothecium formation. Cleistothecium formation through self-crossing was induced by growing the strains on glucose minimal medium (GMM) plates that were sealed airtight and incubated for 14 days at 30 or 37°C. Plates were scanned for the presence of cleistothecia under a light microscope. To assess ascospore viability, five cleistothecia of each strain were collected, cleaned on 4% (wt/vol) agar plates, and resuspended in 100 μl of water. Ascospores were counted, and 100 ascospores were plated on GMM before CFU counts were determined. Cleistothecium density was determined through counting the number of cleistothecia of a certain area and dividing the value by the area (in square centimeters).

Cleistothecium formation through outcrossing was carried out as described previously (57). To induce *pyrG*$^-$ auxotrophy in strains MO80069 and SP-2605-48 (Table 1), they were grown on GMM plates supplemented with 1.2 g/liter uridine and uracil (UU) and 0.75 mg/ml 5-fluoroorotic acid (FOA) in the form of a cross until single colonies appeared. Auxotrophy was confirmed by growing strains on GMM with and without UU before strains were crossed with strain R21XR135 (Table 1).

DNA extraction, genome sequencing, and detection of SNPs and indels. DNA was extracted as described previously (57). Genomes were sequenced using 150-bp Illumina paired-end sequence reads at the Genomic Services Lab of Hudson Alpha (Huntsville, AL). Genomic libraries were constructed with the Illumina TruSeq library kit and sequenced on an Illumina HiSeq 2500 sequencer. Samples were sequenced at greater than 180× coverage or depth.

The Illumina reads were processed with the BBDuk and Tadpole programs of BBMap release 37.34 (https://sourceforge.net/projects/bbmap/files/BBMap_37.34.tar.gz/download) to remove sequencing adapters and phiX and to correct read errors.

Two different Illumina assemblies were performed with the trimmed reads, using platanus (63) and sparseAssembler (64). Nanopore reads were first filtered for quality using Nanofilt (quality of >7) and then were corrected using Canu (65). Once corrected, a subset of reads covering 30 times the estimated genome size of 30 Mb was selected, giving preference to the longest reads. DBG2OLC (66) was used with each of the two Illumina assemblies and the subset of nanopore reads to perform two hybrid assemblies. Independently, MaSuRCA (67) was used to perform a hybrid assembly using the raw nanopore and Illumina reads. The three hybrid assemblies were then corrected using Pilon (68) for three rounds on each

assembly. Ragout (69) was then used to fuse the three assemblies into one final assembly using the assembly obtained with MaSuRCA as the base and the other two as references. This assembly was then corrected again for three rounds using Pilon. The mitochondrial genome was obtained from the discarded contigs of MaSuRCA.

The *Aspergillus nidulans* FGSC A4 genome sequence and gene predictions, were obtained from the Aspergillus Genome Database (version s10-m04-r15 [http://aspgd.org/]). The processed DNA reads were mapped to the FGSC A4 genome with minimap2, version 2.17 (https://github.com/lh3/minimap2), and variants from the FGSC A4 sequence were called with Pilon, version 1.23 (https://github.com/broadinstitute/pilon). Short indels and nucleotide polymorphisms were recovered from the Pilon VCF files by filtering with vcffilter (https://github.com/vcflib/vcflib) to retain only calls with read coverage deeper than 7, exactly one alternative allele, and an alternative allele fraction of at least 0.8. Longer indels and sequence polymorphisms were recovered by searching the VCF files for the SVTYPE keyword. Support for the detected indels was verified by mapping reads to a modified version of the reference genome generated by Pilon. The read coverage depths over inserted sequences were compared to the coverage of flanking sequences, and deletion sites were checked for breaks in read coverage. Sequence variations inside predicted genes and their effects on predicted protein sequence were identified with a custom Python script. The mitochondrial genome was obtained from the discarded contigs of MaSuRCA. Due to its circular nature, the mitochondrial genome appeared repeated multiple times in a single contig. Lastal (http://last.cbrc.jp/doc/last.html) was used to extract one single copy of the mitochondrial genome using the reference mitochondrion.

Detection of large genome deletions and insertions. Genome assemblies of the two clinical isolates were aligned to the FGSC A4 reference genome with nucmer (70). The alignments were filtered to keep only one-to-one matches. Strain-specific loci were detected by searching the alignment coordinates table for regions of the A4 genome with no match in the clinical isolate genome. Large insertions were detected by searching the alignment coordinate table for regions of the clinical isolate genomes with no match in the A4 genome.

Identification of transposon-like regions in the FGSC A4 reference genome. Transposon-like regions were identified by running Pfam (71) on the six translation frames of the complete genome sequence. Regions containing any of the 14 domains typically known to be associated with transposable elements (see Table S1 at the URL mentioned above) were collected. Inverted repeats longer than 50 bp and separated by less than 5,000 bp were extracted and marked as potential miniature inverted-repeat transposable elements (MITE). The Pfam and MITE locations were combined to form the transposon track.

Figure generation. DNAPlotter (72) was used to display the loci of all nonsynonymous SNPs and large deletions identified in the two clinical strains compared to the reference genome of FGSC A4. In addition, the locations of transposon-like regions in the A4 genome were also highlighted using DNAPlotter.

Western blotting. Strains were grown from 1×10^7 spores at 37°C and 200 rpm in 50 ml of CM for 16 h before being exposed to 0.5 M NaCl for 0, 10, and 30 min. Total cellular proteins were extracted according to Fortwendel and colleagues (73) and quantified according to Hartree (74).

For each sample, 60 μg of total intracellular protein was run on a 12% (wt/vol) SDS-PAGE gel before being transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Phosphorylated MpkA or total MpkA was probed for by incubating the membrane with a 1:5,000 dilution of the anti-phospho-p44/42 MAPK (9101; Cell Signaling Technologies) antibody or with a 1:5,000 dilution of the p44-42 MAPK (Cell Signaling Technology) antibody overnight at 4°C, with shaking. Subsequently, membranes were washed three times with TBS-T (2.423 g/liter Tris, 8 g/liter NaCl, 1 ml/liter Tween 20) and incubated with a 1:5,000 dilution of an anti-rabbit IgG horseradish peroxidase (HRP) antibody (7074; Cell Signaling Technologies) for 1 h at room temperature. MpkA was detected by chemiluminescence using a Western ECL Prime (GE Healthcare) blot detection kit according to the manufacturer's instructions. Films were submitted to densitometric analysis using ImageJ software (http://rsbweb.nih.gov/ij/index.html). The amount of phosphorylated MpkA was normalized by the amount of total MpkA. The *A. fumigatus* Δ*mpkA* strain was used as a negative control (Table 1) (75).

Isolation and differentiation of BMDM. Bone marrow-derived macrophages (BMDMs) were isolated as described previously (76). Briefly, BMDMs were recovered from femurs of C57BL/6 wild-type and gp91^{phox} knockout mice and were incubated in BMDM medium (RPMI medium [Gibco] supplemented with 30% [vol/vol] L929 growth-conditioning medium, 20% inactivated fetal bovine serum [FBS; Gibco], 2 mM glutamine, and 100 units/ml of penicillin-streptomycin [Life Technologies]). After 4 days, fresh medium was added for an additional 3 days before BMDMs were collected.

***In vitro* phagocytosis and killing assays.** Phagocytosis and killing assays of *A. nidulans* conidia by wild-type and gp91^{phox} knockout macrophages were carried out according to Bom et al. (77) with modifications. Twenty-four-well plates containing a 15-mm-diameter coverslip in each well (phagocytosis assay) or without any coverslip (killing assay) and 2×10^5 macrophages per well were incubated in 1 ml of RPMI-FBS medium (RPMI medium [Gibco] supplemented with 10% inactivated FBS[Gibco], 2 mM glutamine, and 100 units/ml of penicillin-streptomycin [Life Technologies]) at 37°C in 5% CO_2 for 24 h. Wells were washed with 1 ml of phosphate-buffered saline (PBS) before the same volume of RPMI-FBS medium supplemented with 1×10^6 conidia (1:5 macrophage/conidium ratio) was added under the same conditions.

To determine phagocytosis, macrophages were incubated with conidia for 1.5 h before the supernatant was removed, and 500 μl of PBS containing 3.7% formaldehyde was added for 15 min at room temperature (RT). Sample coverslips were washed with 1 ml of ultrapure water and incubated for 20 min with 500 μl of 0.1 mg/ml calcofluor white (CFW) to stain for the cell wall of nonphagocytized conidia.

Samples were washed, and coverslips were viewed under a Zeiss Observer Z1 fluorescence microscope. In total, 100 conidia were counted per sample, and the phagocytosis index was calculated. Experiments were performed in biological triplicates.

To determine macrophage-induced killing of conidia, macrophages were incubated with conidia for 1.5 h before cell culture supernatants were collected and cytokine concentrations were determined. Macrophages were then washed twice with PBS to remove all nonadherent cells and subsequently lysed with 250 μl of 3% (vol/vol) Triton X-100 for 10 min at RT. Serial dilutions of lysed samples were performed in sterile PBS and plated onto CM and incubated at 37°C for 2 days before CFU counts were determined.

PMN cell isolation and spore germination assay. Human polymorphonuclear (PMN) cells from fresh venous blood of healthy adult volunteers were isolated according to Drewniak et al. (78), with modifications. Cells were harvested by centrifugation in isotonic Percoll, lysed, and resuspended in HEPES-buffered saline solution. *A. nidulans* asexual spores were incubated with PMN cells (1×10^5cells/ml; ratio of 500 PMN:1 conidium) in a 96-well plate overnight at 37°C in RPMI 1640 medium containing glutamine and 10% fetal calf serum (Life). PMN cells were lysed in a solution of water and sodium hydroxide (pH 11.0) (Sigma-Aldrich), and spore germination was determined using an MTT (thiazolyl blue; Sigma-Aldrich) assay. Strain viability was calculated relative to incubation without PMN cells, the level of which was set at 100% for each sample. The viability of *A. nidulans* germinated spores in the presence of PMN cells was determined as described previously (32).

***In vivo* infections in immunocompetent, CGD, and neutrophilic zebrafish.** We evaluated strain virulence in an established zebrafish-aspergillosis model. Seventy-two wild-type larvae were used as an immunocompetent model. Larvae with a dominant negative Rac2D57N mutation in neutrophils (*mpx*::*rac2D57N*) (39) were used as a model of leukocyte adhesion deficiency, where neutrophils do not reach the site of infection, and p22^{phox}-deficient larvae [p22$^{phox\ (sa11798)}$] were used as a chronic granulomatous disease (CGD) model (21).

Spore preparation and conidium microinjection into the hindbrain of 2-day postfertilization (dpf) larvae were performed as previously described (79). Briefly, after manual dechorionation of embryos, 3 nl of inoculum or PBS-control was injected into the hindbrain ventricle via the optic vesicle (~50 conidia) in anesthetized larvae at approximately 36 h postfertilization.

***In vivo* infections in immunocompetent, CGD, and neutropenic mice.** Virulence of the *A. nidulans* strains was determined in immunocompetent, CGD, and neutropenic mice. *A. nidulans* conidial suspensions were prepared, and viability experiments were carried out as described previously (77). Eight- to 12-week-old wild-type ($n = 10$) and gp91^{phox} knockout ($n = 7$) C57BL/6 male mice were used as immunocompetent and CGD models, respectively. Neutropenia was induced in 7- to 8-week-old BALB/c female mice ($n = 10$, weighing between 20 and 22 g) with cyclophosphamide at a concentration of 150 mg per kg, administered intraperitoneally (i.p.) on days -4 and -1 prior to infection (day 0) and at 2 days postinfection. Hydrocortisone acetate (200 mg/kg) was injected subcutaneously on day -3 prior to infection.

Mice were anesthetized and submitted to intratracheal (i.t.) infection as previously described (80) with some minor modifications. Briefly, after i.p. injection of ketamine and xylazine, animals were infected with 5.0×10^7 (immunocompetent) or 1×10^6 (CGD) conidia contained in 75 μl of PBS (81) by surgical i.t. inoculation, which allowed dispensing of the fungal conidia directly into the lungs. Neutropenic mice were infected by intranasal instillation of 1.0×10^4 conidia as described previously (70). Phosphate-buffered saline (PBS) was administered as a negative control for each murine model.

Mice were weighed every 24 h from the day of infection and visually inspected twice daily. The endpoint for survival experimentation was identified when a 20% reduction in body weight was recorded, at which time the mice were sacrificed.

Statistical analyses. All statistical analyses were performed using GraphPad Prism, version 7.00 (GraphPad Software, San Diego, CA), with a P value of < 0.05 considered significantly different. A two-way analysis of variance (ANOVA) was carried out on all stress response tests whereas a one-way ANOVA with Tukey's posttest was applied for growth in the presence of different carbon sources, for the phagocytosis index, and for the PMN cell killing assay. Survival curves were plotted by Kaplan-Meier analysis, and results were analyzed using a log rank test. All experiments were repeated at least twice.

Data availability. Short-read sequences for these strains are available in the NCBI Sequence Read Archive (SRA) under accession numbers SRR10983230, SRR10983231, SRR10983232, and SRR10983233 and BioProject number PRJNA603646. Genomes were deposited in GenBank under accession numbers JAAFYM000000000 and JAAFYL000000000.

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