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Estudo da atividade anti-*Paracoccidioides* do composto galato de decila: mecanismo de ação e interação fungo-hospedeiro

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2016

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Tese apresentada ao Programa de Pós-Graduação em Biociências e Biotecnologia aplicadas à Farmácia da Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista "Júlio de Mesquita Filho" como pré-requisito para obtenção do título de Doutor.

Orientadora: Profa. Dra Maria José Soares Mendes Giannini
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por sempre ter me colocado em primeiro lugar.

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SUMÁRIO

Capítulo I 8
LISTA DE UNIDADES E ABREVIATURAS 9
LISTA DE TABELAS 10
LISTA DE FIGURAS 11
RESUMO 14
ABSTRACT 15
1. INTRODUÇÃO 16
1.1. Nicho ecológico 16
1.2. Estudo filogenético 17
1.3. Paracoccidioidomicose (PCM) 17
1.4. Epidemiologia 20
1.5. Epidemiologia fora da América Latina 21
1.6. PCM *versus* imunodepressão 22
1.7. Fatores que influenciam a infecção 22
1.8. Terapêutica 23
1.9. Antifúngicos de origem natural 24
1.10. Busca de novos fármacos 25
1.11. Gênero *Alchornea* e ácido gálico 26
2. OBJETIVO GERAL 29
3. MATERIAIS E MÉTODOS 30
3.1. Composto com atividade anti-*Paracoccidioides* 30
3.2. Fungo gênero *Paracoccidioides* 30
3.2.1. Meios de cultura 30
3.2.2. Condições gerais 31
3.2.3. Inóculo 31
3.3. Modelo Cultura celular 31
3.3.1. Meio de cultura 31
3.3.2. Condições gerais 32
3.4. Modelo *Galleria mellonella* 32
3.4.1. Condições gerais 32
3.5. Ensaio MTT 33
3.6. Quantificação da concentração de nitritos no sobrenadante da cultura de macrófago RAW após contato com o composto G14. 33
3.7. Análise do composto G14 na atividade mitocondrial e no desenvolvimento das células fúngicas de *P. lutzii.* 34
3.8. Protocolos de marcação 35
3.8.1. Marcação das células fúngicas 35
3.8.2. Marcação das células de mamífero e hemócito 36
3.9. Parâmetros utilizados para análise por citometria de fluxo 36
3.10. Parâmetros utilizados para análise por citometria de imagem 37
3.11.1. Citometria de imagem 39
3.11.2. Citometria de fluxo 39
3.12.1. Citometria de fluxo 40
3.14. Citotoxicidade em cultura celular 41
3.15. Análise da influência do G14 na taxa de interação por citometria de fluxo 41
3.15.1. Interação célula de mamífero e células fúngicas de *P. lutzii* desafiada com o composto G14 41
3.15.2. Interação hemócitos e células fúngicas de *P. lutzii* desafiada com o composto G14 42

3.16. Curva de sobrevivência da larva *G. mellonella* frente ao G14. 42
3.17. Avaliação do mecanismo de ação dos galatos de alquila 42
3.17.1. Ensaio de interação químico-genético..... 43
3.17.1.1. Modelo *Saccharomyces cereviseae*..... 43
3.17.1.2. Teste de sensibilidade..... 43
3.17.1.3. Extração DNA genômico 44
3.17.2. Interação genética..... 45
3.17.3. Análise dos mutantes preditos..... 45
3.17.3.1. Método 50:50 45
3.17.3.2. Cepa GFP x galatos..... 50
3.17.4. Avaliação da n-glicosilação 51
3.17.4.1.1. Preparo da amostra 51
3.17.4.1.2. Avaliação da atividade enzimática 52
3.17.5. Análise da expressão diferencial de genes 52
3.17.5.1. Extração de RNA total 52
3.17.5.2. Síntese de cDNA 53
3.17.5.3. PCR em tempo real 54
4. RESULTADOS 55
4.1. Quantificação da concentração de nitritos no sobrenadante da cultura de macrófago RAW após contato com o composto G14. 55
4.2. Análise do composto G14 na atividade mitocondrial e do desenvolvimento das células fúngicas de *P. lutzii*. 56
4.3. Perfil de interação e influência do composto G14..... 57
4.5. Curva de sobrevivência 61
4.6. Avaliação do mecanismo de ação..... 61
4.6.1. Ensaio químico-genético..... 61
4.6.3. Interação genética 64
4.6.4. Método 50:50..... 66
4.7. Cepa UPR-GFP x galatos 68
4.8. Avaliação da n-glicosilação..... 68
5. DISCUSSÃO..... 71
6. CONCLUSÃO..... 80
7. REFERÊNCIAS 82
Capítulo II..... 99

Capítulo I

LISTA DE UNIDADES E ABREVIATURAS

AIDS: *Acquired Immunodeficiency Syndrome*
ATCC: *American Type Culture Collection*
BHI: *Brain and Heart Infusion*
cDNA: ácido desoxirribonucléico complementar
CFDA-SE*: Carboxyfluorescein diacetate succinimidyl ester*
CIM: concentração inibitória mínima
DAPI: 4',6-Diamidino-2-Phenylindole
DEPC: Dietil pirocarbonato
DMEM*: Dulbecco's Modified Eagle Medium*
DMSO: Dimetilsulfóxido
DO: densidade óptica
DTT: Ditiotreitol
HIV/Aids: *Human Immunodeficiency Virus* / Síndrome da imunodeficiência adquirida
EDTA: *Ethylenediamine tetraacetic acid*
g: gramas
GFP: *Green fluorescent protein*
L: litro
LPS: lipossacarídeo
M: molar
mM: milimolar
mg: miligramas
mL: mililitro
µg: microgramas
µL: microlitro
µM: micromolar
MTT: brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio
NEED: *N-1-naphthyl-ethylenediamine-dichloroplatinum*
nm: nanômetros
Pl01: *Paracoccidioides lutzii* cepa 01
Pb18: *Paracoccidioides brasiliensis* isolado 18
pb: pares de base
PBS: tampão fosfato, pH 7,2
PCM: paracoccidioidomicose
PCR: *Polymerase Chain Reaction*
RNA: ácido ribonucleico
RPMI-1640: *Roswell Park Memorial Institute* medium
RPM: rotações por minuto
TE: solução tampão Tris e EDTA
UPR: *unfold protein response*
°C: graus celsius

LISTA DE TABELAS

Tabela 1: Iniciadores específicos para genes codificantes (F: *foward,* R: *reverse*). 54

Tabela 2: Correlação dos galatos e os genes possivelmente afetados, acompanhados dos processos envolvidos. 65

Tabela 3: Correlação dos agentes utilizados como controle e os genes possivelmente afetados, acompanhados dos processos envolvidos. 66

LISTA DE FIGURAS

Figura 1: Esquema para análise por citometria de fluxo no ensaio de interação com dupla marcação. Equipamento: BD FACS Canto. Software de análise: BD FACS Diva. P1: *gate* população de células de mamífero (macrófagos, linhagens pulmonares) e hemócitos da larva *G. mellonella*. P2: *gate* derivado da população P1, população positiva para a marcação faloidina. P3: *gate* derivado da população P2, positiva para CFDA-SE. 37

Figura 2: Esquema para análise por citometria de imagem no ensaio de interação com dupla marcação. Equipamento: *In Cell Analyzer 2000*. Software de análise: *In Cell Investigator*. As células foram detectadas de acordo com o marcador fluorescente e os parâmetros de tamanho estabelecidos. (A): *wave* 1 – núcleo/DAPI, (B): *wave2:* fungo/ CFDA-SE, (B) *wave* 3: células/ faloidina - Alexa Fluor 647. Circulo azul: detecção da célula; circulo amarelo: detecção do fungo; circulo vermelho (NI): não detecção de interação; circulo verde (I): detecção de interação. 38

Figura 3: A: Região do plasmídeo com a marca de seleção. B: Iniciadores na região homóloga a ser amplificada. C: Fragmento final. 47

Figura 4: Esquema da recombinação da região homóloga do fragmento e do gene no genoma, levando a integração do fragmento com uma marca de seleção (imagem: (Horecka e Davis, 2014). 48

Figura 5: Esquema recombinação entre uma região do fragmento inserido e uma região homóloga no DNA genômico. 49

Figura 6: Esquema para análise das cepas GFP. 51

Figura 7: Avaliação do potencial antioxidante do G14: (A/B) monocamada de macrófago peritoneal murino RAW desafiada com G14 na faixa de concentração de 1 a 0,13 µg/mL. (A) com estimulação de LPS (1 mg/ml), (B) sem estimulação por LPS. Barra branca: macrófagos não tratados com G14 e não estimulados com LPS. Barra preta: macrófagos não tratados com G14 e estimulados com LPS (1 mg/ml). (C) Taxa de viabilidade da linhagem de macrófago peritoneal murino RAW após 24 horas de contato de G14 na faixa de concentração de 1 a 0,13 µg/mL . *em relação ao controle Meio ($p<0,05$); # em relação ao controle de LPS ($p<0,05$). (ANOVA/Tukey's post-test). 56

Figura 8: Análise da atividade mitocondrial e do desenvolvimento das células fúngicas de *P. lutzii* após 24 e 48 horas de contato com as concentrações de 0,5 a 0,13 µg/mL do composto galato de decila. A: ensaio de MTT para análise da atividade mitocondrial; B: porcentagem de células com brotamentos para analise do desenvolvimento. Barra controle: células fúngicas avaliadas sem contato com G14 em cada tempo. *$p<0,05$ em relação ao controle do tempo. # $p<0,05$ em relação à condição tratado 0,5 µg/mL. (ANOVA/Tukey's post-test). 57

Figura 9: Perfil de interação das células fúngicas de *P.lutzii* com as linhagens pulmonares humanas A549 (A, B) e MRC5 (C, D), a linhagem de macrófago alveolar murino AMJ2-C11 (E) e hemócitos da larva de *G. mellonella* (F) nos tempos de 1, 3, 6 e 24 horas por citometria de imagem (A,C) e de fluxo (B,D,E,F). Barra T: Taxa de interação após 24 horas de tratamento com G14 na concentração de 0,5 µg/mL por citometria de fluxo em comparação com a taxa de interação do tempo de 24 horas (B,D,E,F). *p<0,05 entre os tempos..... 58

Figura 10: Análise das imagens adquiridas no equipamento *In Cell Analyzer* 2000 da interação das células fúngicas de *P. lutzii* (Pl01) as linhagens de pulmonares humana A549 e MRC-5; e aos hemócitos da larva de *G. mellonella* após 6 horas de interação. (A) interação Pl01 e MRC-5; (B) interação Pl01 e A549; (C) interação Pl01 e hemócito. Seta branca: internalização do fungo ao tipo celular estudado; seta vermelha: fungo aderido ao tipo celular estudado. Células fúngicas marcada com CFDA-SE 15 µM (vermelho) e linhagem pulmonar e hemócitos marcados com Alexa Fluor® 647 faloidina 0,165 µM (verde). (A e B) núcleo das linhagens pulmonares marcado com DAPI..... 60

Figura 11: Estudo da sobrevivência de *Galleria mellonella* após infecção com Pl01 e tratamento com G14 durante 10 dias de análise. Grupo controle PBS (linha preta), grupo infectado Pl01 (linha vermelha), grupo infectado Pl01 por seis horas e posteriormente tratado com dose equivalente a 0,5 µg/mL de G14 (linha verde), grupo de larvas inoculado com dose equivalente a 0,5 µg/mL de G14. * p<0,05 em relação ao grupo Pl01 (infectado). 61

Figura 12: Figura representativa da hipersensibilidade da coleção aos galatos em diferentes concentrações. A escala de cores indica a variedade da porcentagem de crescimento. VERDE: porcentagem de crescimento semelhante ao controle DMSO; VERMELHO: porcentagem de crescimento baixa em relação ao controle DMSO. (A): ácido gálico e os derivados hexila, heptila, nonila, decila, undecila, dodecila e tetradecila testados na faixa de concentração de 100 a 0,75 µM. (B): galatos selecionados nonila, decila, undecila e dodecila testados na faixa de concentração de 50 a 12,5 µM. Em destaque concentração selecionada: 22,5µM..... 62

Figura 13: A: Gel 2 % agarose para a confirmação do produto de PCR de cada condição contendo *barcode* do mutante mais a integração do marcador individual 10 pb. Tamanho produto final 267 pb. B: Padrão de 250 a 10000 pb (ThermoFicherScientific-SM1163). C: Exemplo gel 2 % agarose para extração da banda com 267 pb contendo a biblioteca de DNA. 63

Figura 14: Gráfico *Java Treeview heatmap*: mutantes coleção *Mini-pool* verso agentes controles, compostos testes e DMSO controle. Vermelho: mutantes hipersensíveis ao tratamento. Verde: mutantes hiper-resistentes ao tratamento. Cinza: sem valor..... 65

Figura 15: (A): Produto de PCR: fragmentos PDR1, PDR3 e SNQ2. (B): confirmação para a inserção do fragmento (*pop-in*), positivo: colchete, negativo: seta vermelha. (C): confirmação para a deleção do fragmento inserido no gene alvo (*pop-out*), positivo: colchete, negativo: seta vermelha. Foi utilizado o padrão de 250 a 10000 pb (ThermoFicherScientific-SM1163)..... 67

Figura 16: Análise do estresse ao retículo endoplasmático pelos galatos de nonila, decila, undecila e dodecila. Cepa da levedura *Saccharomyces cerevisiae* com sequencia GFP inserida próxima aos genes do grupo UPR, foi tratada com galatos de nonila, decila, undecila e dodecila na concentração de 22,5 μM por 3 e 24 horas. Por microscópio de fluorescência foi observado a ativação ou não grupo UPR através da fluorescência do grupo GFP expresso concomitantemente. 68

Figura 17: Análise da influência do galato de decila (G14) na atividade relativa de enzimas n-glicosiladas. Células fúngicas das espécies *P. lutzii* (Pl01) e *P. brasiliensis* (Pb18) foram desafiadas com o G14 na concentração de 0,5μg/mL (+/barra preta) por 72 horas e a porcentagem da atividade relativa das enzimas α-(1,3)- glucanase, N-Acetyl-β-D-glucosaminidase e α-amilase foi avaliada de acordo com o controle sem tratamento (-/barra branca). *p<0,05 em relação ao controle..... 69

Figura 18: RNA total submetido a gel de agarose 1 %. Pl01: controle (C) e tratado (T); Pb18: controle (C) e tratado (T). setas: bandas RNAr 26S e 18S, respectivamente. 70

Figura 19: Análise por PCR em Tempo Real da expressão relativa dos genes que codificam α-(1,3)-glucanase (AGN), α-(1,3)-glucano sintase (AGS), N-Acetyl-β-D-glucosaminidase (NAG), α-amilase (AMY), e os genes sensores de estresse ao retículo endoplasmático IRE1 e HAC, nas células fúngicas das espécies *P. lutzii* (Pl01) *e P. brasiliensis* (Pb18) desafiadas com o galato de decila (G14) na concentração de 0,5μg/mL por 72 horas. Método utilizado para análise: $2^{-\Delta\Delta CT}$. Controle gene endógeno α-tubulina. Controle negativo: amostra substituída por água DEPC. *p<0,05 em relação ao gene da outra espécie. 71

RESUMO

O fungo do gênero *Paracoccidioides* é o agente etiológico da paracoccidioidomicose (PCM), micose de grande importância no Brasil e América Latina. Há diferentes terapias disponíveis, como os antifúngicos da classe dos poliênicos, azóis e sulfonamidas. O desenvolvimento de novos antifúngicos com eficácia superior é necessário para prevenir recaídas e diminuir os efeitos secundários, o período de tratamento e sequelas. A molécula de ácido gálico com diferentes modificações (galatos de alquila) apresentou atividade antifúngica contra um painel representativo de fungos patogênicos. O galato de decila (G14) foi objeto de estudo deste trabalho, para o qual a interação *Paracoccidioides*-hospedeiro foi relacionada ao mecanismo de ação. Para tanto, o fungo *Paracoccidioides lutzii* (Pl01) foi desafiado com o G14 para análise das alterações no desenvolvimento de brotamentos e na viabilidade celular pelo ensaio de MTT. Foi avaliada a influência do G14 na taxa de interação de Pl01 com a linhagem de macrófago alveolar murino AMJ2-C11, com as linhagens pulmonares de mamífero A549 e MRC-5, e hemócitos da larva de *Galleria mellonella* utilizando ensaios por citometria de fluxo. O G14 ainda foi avaliado quanto à taxa de sobrevivência da larva de *G. mellonella* infectada e a capacidade na modulação da produção de óxido nítrico em macrófago RAW. O estudo do mecanismo de ação foi realizado pelo ensaio de interação químico-genética seguida da interação genética, que consiste na comparação de uma coleção de mutantes da levedura *Saccharomyces cereviseae* com perfil hipersensível ao G14, a um banco de dados de duplos mutantes para predizer a via afetada. Em *P. lutzii* (Pl01) e *P. brasiliensis* (Pb18) foi avaliada a taxa de atividade de enzimas n-glicosiladas (α-(1,3)-glucanase, N-Acetyl-β-D-glucosaminidase e α-amilase) ligadas à formação da parede celular; assim como a expressão relativa dos genes envolvidos através de PCR em tempo real. No ensaio de interação químico-genética, cerca de 50 mutantes foram hipersensíveis e com a análise da interação genética deduziu-se influência sobre a via de glicosilação e/ou na resposta a proteínas não enoveladas. A concentração de 0,5 µg/mL de G14 apresentou redução significativa de 42 % da atividade metabólica e de 38,5 % na formação de novos brotos do Pl01 com 24 horas de contato, indicando forte influência sobre o desenvolvimento da célula fúngica. As enzimas n-glicosiladas testadas para Pl01 e Pb18 apresentaram baixa atividade quando desafiadas com G14, porém os genes referentes a estas enzimas não apresentaram expressão relativa baixa, inferindo a influência nas modificações pós-traducionais destas enzimas. A influência do G14 na atividade funcional destas enzimas resulta na instabilidade da parede celular, a qual é um elemento crucial para a virulência e para a interação *Paracoccidioides*-célula hospedeira. Assim, foi observada redução na taxa de interação do Pl01 de 1,6 vezes com a linhagem A549, e de 1,2 com a linhagem AMJ2-C11. Além disso, foi observado um aumento no tempo de sobrevivência da larva infectada com Pl01 e tratada, indicando que G14 tem propriedades benéficas ao hospedeiro, o que foi observado com a redução de 20 % na quantificação de óxido nítrico quando macrófagos RAW estimulados por LPS foram desafiados com G14, o que sugere uma atividade antioxidante.

Palavras-chave: Galato de decila. Óxido nítrico. Citometria de fluxo. Interação químico genética. n-glicosilação.

ABSTRACT

The fungus of genus *Paracoccidioides* is the etiological agent of paracoccidioidomycosis (PCM), an important mycosis in Brazil and Latin America. There are different available therapies, as antifungals belong to the class of polyenes, azoles and sulfonamides. The development of new antifungal agents with greater effectiveness is required to prevent relapse and reduce side effects, the period of treatment and sequelae. The gallic acid molecule with different modifications (alkyl gallates) showed antifungal activity against a representative panel of pathogenic fungi. The decyl gallate (G14) was the study object of this work, for which the *Paracoccidioides*-host interaction was connected with the mechanism of action. Therefore, the fungus *Paracoccidioides lutzii* (Pl01) was challenged with the G14 to analyze the changes in the development of budding and cell viability by MTT assay. Then, it was assessed the influence of the G14 in the interaction rate of Pl01 with murine alveolar macrophage lineage AMJ2-C11, with mammalian lung lineage A549 and MRC-5, and hemocytes of *Galleria mellonella* larvae using assays by flow cytometry. The compound G14 was evaluated for survival rate of infected *G. mellonella* larvae and to capacity of modulation of nitric oxide production in RAW macrophages. The mechanism of action was performed by chemical-genetic interaction assay followed by genetic interaction, which involves comparing a mutant collection, of *Saccharomyces cerevisiae* yeast, with hypersensitive profile to G14 to double mutant database to predict via affected. In *P. lutzii* (Pl01) and *P. brasiliensis* (Pb18) was evaluated the rate of activity of n-glycosylated enzymes (α- (1,3) -glucanase, N-Acetyl-β-D-glucosaminidase and α-amylase) linked to cell wall formation; as well as the relative expression of the genes involved by real time PCR. In the test chemical-genetic interaction, about 50 mutants were hypersensitive to G14 and with the genetic interaction analysis was deduced that it influence the glycosylation pathway and/or in the response unfolded proteins. The concentration of 0.5 μg/ml of G14 after 24 hours of contact showed a significant reduction of 42% in metabolic activity and 38.5% in the formation of new buds of Pl01, indicating a strong influence on the development of the fungal cell. The N-glycosylated enzymes tested for Pl01 and Pb18 after 72 hours of contact with 0.5 μg/ml of G14 showed low activity when challenged with G14, but the genes related to these enzymes did not show low relative expression, inferring the influence on the post-translational modification of these enzymes. The influence of the G14 in the functional activity of these enzymes results in the instability of the cell wall, which is crucial to the virulence and the host *Paracoccidioides*-cell interaction. Thus, it was observed a reduction in the interaction rate of Pl01 in 1.6 times with lineage A549 and in 1.2 times with lineage AMJ2-C11. Besides that, it was observed an increase of survival time of Pl01infected larvae and treated, indicating G14 has properties that benefit the host, what was observed with a reduction of 20% in the quantification of nitric oxide when RAW macrophages stimulated by LPS were challenged with G14, which showed an antioxidant activity.

Keywords: Decyl gallate. Nitric oxide. Flow cytometry. Chemical-genetic interaction. N-glycosylation.

1. INTRODUÇÃO

Paracoccidioides spp é o agente etiológico da paracoccidioidomicose (PCM), micose de alto impacto social e médico em áreas endêmicas, devido ao número de casos, e por ser doença crônica, com um tratamento de longa duração e alta taxa de sequelas, as quais podem causar inabilidade ao trabalho físico e uma baixa qualidade de vida. Além disso, o fato da notificação não ser obrigatória, a ausência dos registros de surtos epidêmicos, a extrema dificuldade no diagnostico precoce aliado à falta de capacidade de um diagnostico laboratorial eficiente, dificulta uma abordagem epidemiológica 100 % realista da doença (MARTINEZ, 2015).

1.1. Nicho ecológico

Pouco se sabe sobre o habitat do *Paracoccidioides* spp. porém alguns estudos na literatura tentam entender a pato-fisiologia deste agente com as informações do seu possível habitat (RESTREPO et al., 2001).

Em uma revisão publicada por Franco et al (2000), foram analisados diferentes publicações que relatavam tentativas de isolar o fungo do solo ou matérias contaminados com solo, sendo que na maioria dos isolados foram confirmados com a caracterização morfológica do fungo, além da avaliação da virulência e da antigenicidade da cepa, e por análises bioquímicas e moleculares.

Evidências epidemiológicas indicam que a forma saprofítica do fungo pode ocorrer em algumas condições restritas do solo, como em locais de vegetação natural, perto de fontes de água ou em áreas com taxas variando de alta a moderada de níveis de precipitação com temperaturas amenas. Bruscas alterações nas condições climáticas que resultem no aumento atípico do armazenamento de água no solo e na taxa de umidade do ar podem contribuir primeiramente para o crescimento do fungo, e posteriormente para a dispersão de conídios (TEIXEIRA et al., 2014). As zonas caracterizadas como endêmicas são comumente as áreas rurais ou de intensa atividade agrícola. O contínuo desmatamento, a extensiva e contínua agricultura, a migração humana para regiões pouco habitadas e inexploradas, com urbanização em condições inadequadas e o impacto ambiental das grandes construções tem um grande impacto sobre a aquisição da PCM (MARTINEZ, 2015).

Há alguns estudos sobre achados deste fungo em animais selvagens, como o tatu, e animais domésticos como em cachorros (BAGAGLI et al., 2006b).

1.2. Estudo filogenético

Em 1908, Adolpho Lutz publicou a descrição no Brasil de uma micose pseudococcídica localizada na boca, denominando as micoses causadas por *Pseudococcidia* como Hifoblastomicoses. Em 1912, Alfonso Splendore isolou o mesmo fungo, classificando-o como *Zymonema brasiliensis*. Posteriormente, em 1930, Almeida após comparações com *Coccidioides immitis,* caracterizou o fungo como do gênero *Paracoccidioides* e da espécie *brasiliensis.* Com base em recentes análises filogenéticas, *Paracoccidioides* spp está classificado na Ordem *Onygenales*, na Família *Ajellomycetaceae* (*Ascomycetes*), formando um clado monofilético que inclui os gêneros *Blastomyces*, *Emmonsia* e *Histoplasma*, com *Lacazia loboi* como ancestral comum (BENCHIMOL; SÁ, 2004; UNTEREINER *et al.*, 2004).

Mais recentemente, os diferentes isolados foram classificados em quatro diferentes espécies filogenéticas, S1, PS2, PS3 e PS4 sendo a espécie S1, um grupo parafilético encontrado no Brasil, Argentina, Paraguai, Peru e Venezuela; a espécie PS2 é um grupo monofilético encontrado no Brasil e Venezuela; a espécie PS3 um grupo monofilético encontrado apenas na Colômbia e a espécie PS4 na Venezuela (MATUTE; MCEWEN, et al., 2006; MATUTE; SEPULVEDA, et al., 2006). Posteriormente, com base na comparação de sequências codificantes e não codificantes de diferentes genes, um isolado denominado *Paracoccidioides lutzii* foi classificado e separado dos demais, e proposta a classificação deste como uma nova espécie filogenética (CARRERO et al., 2008). Contudo, foi estimado que as espécies filogenéticas S1/PS2/PS3/PS4 e *P. lutzii* tiveram um ancestral comum a cerca de 32 milhões de anos (TEIXEIRA et al., 2009). A região centro-oeste do Brasil é considerada a de maior prevalência dos casos de PCM acometidos pela espécie *P. lutzii* (MARQUES-DA-SILVA et al., 2012). A correta taxonomia molecular deste fungo tem aberto novas possibilidades para o estudo e o entendimento de suas relações eco epidemiológicas com seus hospedeiros (BAGAGLI et al., 2006a; BAGAGLI et al., 2008; TEIXEIRA et al., 2014; NOBREGA DE ALMEIDA et al., 2015).

1.3. Paracoccidioidomicose (PCM)

- *Rota de infecção*

O gênero *Paracoccidioides* é formado por fungos termodimórficos, os quais apresentam a forma miceliar a 25-27 °C (forma saprofítica-infectante) e forma leveduriforme a 35- 37 °C (forma parasitária). Devido a intensa atividade agrícola presente

nas áreas endêmicas, conídios suspensos no ar, pertencentes à fase miceliar do fungo do gênero *Paracoccidioides,* são inalados pelo hospedeiro. Estes invadem as vias aéreas inferiores, atingindo os alvéolos e bronquíolos terminais. Neste local, a temperatura corporal influencia na diferenciação celular transformando-se na forma leveduriforme multinucleada (RESTREPO, 1985; SAN-BLAS et al., 1993; LACAZ, 1994).

Uma vez inalados, os conídios do fungo entram em contato com o aparato pulmonar e desencadeiam uma reação inflamatória, com presença de macrófagos, células gigantes multinucleadas, linfócitos e células epitelióides formando um granuloma. Na área central do granuloma é comum a presença de linfócitos e macrófagos, os quais por meio da produção de citocinas e outros mediadores inflamatórios assumem importância central na morfogênese e na formação e modulação do granuloma (FORTES et al., 2011). Desta forma, a PCM é considerada uma doença granulomatosa com diferentes formas clínicas. Em pacientes com resposta imune celular preservada, observam-se granulomas epitelióides compactos, bem definidos, com poucos fungos. Aqueles indivíduos com comprometimento imunológico, a inflamação granulomatosa é desorganizada, com grande número de fungos e granulomas frouxos, mal definidos, nestes casos ocorre a proliferação e disseminação do fungo, levando à doença generalizada de mau prognóstico (NISHIKAKU et al., 2009; FORTES et al., 2011).

- *Sistema imunológico*

Tem sido relatado que um elevado nível da resposta imune humoral (Th-2) está associado com o aumento da disseminação da doença, envolvendo IL-4, IL-5 e TGF-β. Por outro lado, a resposta imune mediada por células (Th-1) representa o principal mecanismo de defesa na PCM, atuando principalmente TNF-α e IFN-γ. Dessa forma a inibição da imunidade Th-2 e a estimulação da imunidade Th-1 geralmente aumenta a eficácia dos antifúngicos utilizados na terapia (CALICH et al., 1987; DE ALMEIDA et al., 1998; BENARD et al., 2001; MAMONI et al., 2002; CALVI et al., 2003; SHIKANAI-YASUDA et al., 2006a; TRAVASSOS; TABORDA, 2012b). Pacientes infectados, classificados como assintomáticos, apresentam uma resposta predominante Th1, enquanto que pacientes com a forma crônica da doença, frequentemente adultos, apresentam padrão Th17/Th22, e na forma aguda (juvenil) uma resposta tipo Th2/Th9 (DE CASTRO et al., 2013; FERNANDES et al., 2015).

- *Formas clínicas*

Entre 1983 a 1986 um grupo de especialistas classificou e determinou as formas clínicas da PCM, as quais foram devidamente publicadas em 1987 por Franco e colaboradores baseando-se no período de latência, da localização e gravidade das lesões, acompanhado do estado nutricional e disfunção do órgão acometido (FRANCO et al., 1987).

Assim, a forma aguda/subaguda, considerada tipo juvenil, representada por cerca de 5 % dos casos, pode ser classificada como moderada ou severa. Geralmente, acomete crianças, adolescentes e adultos com até 35 anos, sem distinção de gênero. Estes pacientes trazem como sintomas febre, perda de peso, e anemia de branda a moderada por 2-3 meses ou mais. Comumente ocorre o expressivo aumento dos nódulos cervicais, axilares e inguinais, assim como do fígado e do baço. Uma alta taxa de pacientes apresenta múltiplas lesões na pele e na mucosa, comprometimento do trato digestivo e há diferentes relatos do acometimento do tecido ósseo (BOCCA et al., 2013).

A forma crônica, com representatividade de mais de 90 % dos casos, ocorre geralmente em indivíduos, com maior frequência do gênero masculino, entre 30 a 60 anos com atividades laborais envolvendo áreas agrícolas. Pode ser classificada de acordo com o tipo de lesão, unifocal ou multifocal. As lesões unifocais estão associadas com a alta severidade da doença, em alguns casos quando acometem o sistema nervoso central e outras funções vitais concomitantemente. As lesões multifocais estão relacionadas com a disseminação da doença. O pulmão é o órgão mais afetado, seguido por lesões na pele e na mucosa (oral, laringe, traqueia, trato digestivo). As lesões cutâneas podem ser únicas ou múltiplas, e são observadas como pápulas ou úlceras principalmente na face. As lesões da mucosas características são ulcerações de fundo granuloso e pontilhado hemorrágico, chamadas de estomatite moriforme. Nódulos linfáticos, glândulas adrenais e outros órgãos podem ser acometidos, com uma menor frequência do intestino, sistema nervoso central, tecido ósseo, baço, olhos, sistema geniturinário e cardiovascular. O curso da doença nesta forma é insidioso, a manifestação de febre é incomum, e quando presente há uma associação com tuberculose. Os pacientes podem demonstrar fraqueza, perda de peso, dispneia, tosse, e às vezes expectoração purulenta e raramente com sangue (BOCCA et al., 2013).

1.4. Epidemiologia

A PCM é uma doença considerada geograficamente limitada à América Latina, com regiões de maior endemicidade, como Brasil, Colômbia e Venezuela, seguidas por outras áreas, de menor incidência, como Bolívia, Paraguai, Argentina, Uruguai e regiões da América Central (SHIKANAI-YASUDA et al., 2006b; AMEEN et al., 2010; DE ABREU SILVA et al., 2012). Em uma revisão foi descrito que 80 % dos casos de PCM foram reportados no Brasil, nos estados de São Paulo, Paraná, Rio Grande do Sul, Rio de Janeiro e Rondônia (COLOMBO et al., 2011).

No período de 1980 a 1995 verificou-se uma média anual de mortalidade por PCM no Brasil de 1,45 por milhões de habitantes, com 3181 mortes (COUTINHO et al., 2002). Acredita-se que sua incidência em zonas endêmicas varie de 3 a 4 novos casos/milhão até 1-3 novos casos a cada 100.000 habitantes ao ano (SHIKANAI-YASUDA et al., 2006a).

As áreas endêmicas do Brasil podem ser divididas de acordo com o grau de incidência da doença, sendo as áreas das regiões Sudeste (estados de São Paulo, Rio de Janeiro, Espírito Santo e Minas Gerais), Centro Oeste (principalmente os estados de Goiás e Mato Grosso do Sul) e Sul (estado do Paraná ao Norte do Rio Grande do Sul) classificadas como altamente endêmicas. As regiões localizadas ao longo da extremidade Leste da Amazônia, incluindo os estados do Pará, Maranhão e Tocantins são classificados como áreas com menor índice da doença. Com taxa de registros ainda menores se destacam as regiões do extremo oeste da região amazônica e o estado de Rondônia, as quais se tornaram relevantes no começo do século 20. Em contraste, a região Nordeste do Brasil é considerada com baixa endemicidade, e a maioria dos casos registrados são advindos de outras regiões (COUTINHO et al., 2002; MARTINEZ, 2015).

Podemos encontrar na literatura trabalhos com estudos e levantamentos regionais. Em um estudo realizado no período de 1953 a 1978, 212 municípios do estado de São Paulo (regiões norte e central do estado) foram relatados por apresentarem 937 casos de PCM, ou seja, uma incidência de 0,3 a 0,4 casos por 100.000 habitantes por ano (FAVA et al., 1987). Entre 1960 a 1999 estimaram uma media de 2,7 casos por ano na cidade de Ribeirão Preto (estado de São Paulo) e pela análise, de 1000 pacientes acometidos, 497 são naturais da cidade (BELLISSIMO-RODRIGUES et al., 2011), e no período de 1970 e 2009 foi registrado um número significativo de 1219 pacientes (BELLISSIMO-RODRIGUES ET AL., 2013), classificando a região como endêmica. Na região da cidade de Campinas (sudeste do Brasil), entre 1988–1996, foram analisados diferentes casos com

suspeita de PCM, sendo então que 89,7% foram confirmados para a doença (Blotta et al., 1999).

Saindo do estado de São Paulo no período de 1958 a 1987, foram notificados 260 pacientes, naturais do estado do Rio grande do Sul, acometidos pela PCM, revelando uma incidência de 0,9 casos por 100.000 habitantes, por ano (Londero e Ramos, 1990). Outro estudo realizado no estado do Rio Grande do Sul, analisou 123 casos de PCM entre os anos de 1966 e 2009, confirmando o interior do estado como uma região endêmica (De Souza et al., 2014). No estado do Paraná no período de 1980 a 1998 um estudo de 551 óbitos por PCM notificados em 184 municípios, com maior número nas regiões Norte, Central e Oeste do estado, revelou uma taxa de mortalidade de 3,48 por milhão de habitantes, (BITTENCOURT et al., 2005). No estado do Maranhão, entre os anos de 1997 e 2007 foram catalogados 216 casos, concluindo que as regiões Oeste, Leste e Centro do Maranhão registraram 90,3% destes casos, sendo que a região Oeste apresenta prevalência de 10,8 casos por 100.000 habitantes (MATOS et al., 2012). No estado de Mato Grosso do Sul entre 1980 a 1999 foi realizado um estudo clínico e epidemiológico de 422 casos demonstrando a região como endêmica (PANIAGO et al., 2003). Em outro estudo foram investigados a incidência e eventos de morte entre 102 portadores de PCM residentes na região do Lago de Itaipu, na Cidade de Foz do Iguaçu (Oeste do Paraná) no período de 2008 a 2009, dos quais 15 vieram a óbito no período do estudo, classificando a região como endêmica (Loth et al., 2011). Uma análise de 2163 pacientes no período de 1997-2012 no estado de Rondônia que com uma media anual de 9,4 casos a cada 100.000 habitantes constatou alta incidência de PCM (VIEIRA et al., 2014).

1.5. Epidemiologia fora da América Latina

Ao menos 60 casos de PCM foram notificados fora da América Latina, como nos Estados Unidos, Canadá, Espanha e outros países europeus. No Japão foram identificados pelo menos 17 pacientes e uma tendência ao aumento do número anual. Novos casos foram observados na Alemanha, Inglaterra, Espanha, França, Holanda e Japão, nos quais os pacientes tinham relatado visitar ou trabalhar em um ou mais países da América do Sul ou Central, incluindo o Brasil, Venezuela, Bolívia, Equador, Argentina e Paraguai. Em alguns relatos, os pacientes já apresentavam manifestações clínicas da PCM logo após deixar os locais endêmicos, porém em sua maioria, apresentaram lesões depois de pelo menos cinco anos de permanência em países não endêmicos, indicando sítios endêmicos da micose (MANNS et al., 1996; TELES; MARTINS, 2011; MARTINEZ, 2015).

1.6. PCM *versus* imunodepressão

Há relatos na literatura do comportamento oportunista do fungo *Paracoccidioides* em pacientes com a resposta imune celular deficiente, através da depressão do sistema imunológico por algum tipo de doença, como a AIDS (*Acquired Immunodeficiency Syndrome*) ou por alguma terapia envolvida. Pacientes imunodepremidos apresentam um quadro de PCM de forma mais disseminada, com uma alta taxa de mortalidade precoce e menor porcentagem de cura depois da terapia antifúngica. Como exemplo, a co-infecção pelo fungo *Paracoccidioides* e o HIV (*human immunodeficiency vírus*) (PANIAGO *et al.*, 2005; MOREJÓN *et al.*, 2009; RIBEIRO *et al.*, 2009), a qual representa, só no Brasil, aproximadamente 1,4 % dos casos de co-infecção fungo/HIV (PRADO *et al.*, 2009). Martinez (2015) cita diferentes exemplos de casos graves de pacientes acometidos pela PCM e imudeprimidos, como por doenças hematológicas ou neoplasia de órgãos sólidos, assim como pacientes submetidos a transplante renal, deficientes de CD40L e com polimorfismo genético dos receptores da interleucina 12. Em todos os casos, é comum a reincidência da PCM decorrentes de lesões residuais, levando a reativação do fungo, culminando no estado grave do paciente (PRADO *et al.*, 2009).

1.7. Fatores que influenciam a infecção

Em adição ao tipo de resposta imunológica e a área que o paciente reside, outros fatores podem influenciar na progressão da doença. A idade é considerada um importante fator, uma vez que há uma maior incidência em pacientes entre 30 e 60 anos, fase na qual o paciente é mais exposto ao fungo devido ao maior envolvimento com atividades profissionais. Estudos demonstram que a forma aguda/subaguda esta associada as três primeiras décadas de vida, e é a única forma que afeta crianças com até 15 anos de idade. A forma crônica é, geralmente, encontrada em pacientes com mais de 30 anos. A taxa de produção do hormônio beta-estradiol, também, é um fator que pode determinar a infecção, uma vez que este inibe o desenvolvimento dos conídios inalados para a forma leveduriforme, além de modular a resposta imune celular. O risco de infecção, também, esta associado em mulheres grávidas, uma vez que na gravidez ocorre a redução temporária da resposta celular. Algumas ações associadas ao estilo de vida tem impacto importante para o desenvolvimento da doença. Dessa forma é observado que o risco é 14 vezes maior para fumantes e 3,6 vezes maior entre indivíduos que consomem quantidades superiores a 60 mL por dia de bebidas destiladas. A dificuldade de cura é maior naqueles

pacientes com hábito de consumo médio de 100 mL/dia destas bebidas (AMEEN et al., 2010; Barrozo et al., 2010; TELES; MARTINS, 2011; MARTINEZ, 2015).

1.8. Terapêutica

Há diferentes antifúngicos que podem ser utilizadas para o tratamento da PCM. Ribeiro em 1940 sugeriu um tratamento inicial com sulfapiridina. Posteriormente, Lacaz e Sampaio propuseram o uso da anfotericina B em 1958. Barbosa e Vasconcelos, em 1973, recomendaram o uso da combinação de trimetoprim-sulfametoxazol. Por volta de 1979, Negroni sugeriu o uso de cetoconazol. Restrepo, em 1987 recomendou itraconazol e mais recentemente, em 2007, o uso de voriconazol foi indicado por Queiroz-Telles (CAVALCANTI LUNA et al., 2015).

O tratamento depende da gravidade da doença, do tipo de agente antifúngico disponível e do tempo de uso. Apesar das informações limitadas sobre as diferentes terapias, o itraconazol é a primeira escolha para controlar as formas clínicas classificadas de brandas a moderada da doença. Desde 1987, muitos grupos desenvolveram estudos com os antifúngicos da classe dos azóis, os quais mostraram uma importante redução nos sintomas e um retardo na progressão da doença (NEGRONI; FINQUELIEVICH, et al., 1987; NEGRONI; PALMIERI; KOREN, et al., 1987; NEGRONI; PALMIERI; CHÁ TOREA, 1987; Restrepo et al., 1987). Porém, a terapia com itraconazol não está facilmente disponível nas áreas endêmicas, o que consequentemente, confere a combinação da terapia trimetoprim-sulfametoxazol (doses diárias por 12 meses nos casos brandos e 24 meses nos moderados) como uma opção mais acessível e mais utilizada. Nos casos em que a PCM atinge o sistema nervoso central o tratamento deve ser conduzido com fluconazol ou voriconazol, diariamente por 3 a 6 meses, pois ambos possuem uma boa penetração através da barreira hematoencefálica (FERREIRA, 2009; MARQUES, 2012). Atualmente, cetoconazol é o menos usado para o tratamento desta infecção, devido aos seus severos efeitos colaterais (hepatoxicidade, perda de libido, inibição da produção de corticoide). Deve ser ressaltado a possibilidade dos derivados triazólicos interagirem com outros fármacos, como os antihistamínicos, antiácidos, bloqueadores dos receptors H2, barbituricos, ciclosporinas, difenilhidantoinícos, digoxinas, cisaprida e rifampicina (FERREIRA, 2009).

Nos casos considerados severos, a terapia com anfotericina B é a primeira escolha, porém são bem conhecidos os efeitos colaterais e tóxicos (nefrotoxicidade, toxicidade miocardical, mielotoxicidade etc) relatados em relação a esse antifúngico, o que pode

acarretar na descontinuação da terapia. Nos últimos 30 anos houve um grande esforço para melhorar a formulação da anfotericina B, como exemplo na forma deoxicolato considerada principal opção no tratamento intravenoso dos casos severos de PCM (PEÇANHA *et al.*, 2016), porém esta terapia é considerada pouco acessiva uma vez que estas fomulações envolvem alto custo. (LORTHLARY et al., 1999; LANIADO-LABORÍN; CABRALES-VARGAS, 2009).

Na literatura não há muitos relatos sobre resistência de *Paracoccidioides* spp. as terapias antifúngicas, no entanto há relatos de que pacientes acometidos pela espécie *P. lutzii* são mais sensíveis a terapia com cetoconazol e trimetoprim-sulfametoxazol do que aqueles acometidos pela espécie *P. brasiliensis* (HAHN et al., 2003). De outra maneira, um estudo demonstrou que o processo de melanização que este fungo é capaz de desenvolver, diminui a sensibilidade a alguns agentes antifúngicos, em particular a anfotericina B (DA SILVA et al., 2006).

As equinocandinas foram descobertas em 1970 através da pesquisa randomizada de metobólicos secundários. É considerada a mais nova classe de agentes antifúngicos, as quais são inibidores da síntese de β-(1,3)-glucano. A caspofungina foi a primeira a ser aprovada para o uso clínico, sendo que as principais descrições na literatura envolvem o tratamento para candidíase e aspergilose (WALKER et al., 2010). Não há relatos do tratamento clínico de PCM com caspofungina, porém estudos *in vitro* avaliaram a sensibilidade de *P. brasiliensis* (fase miceliar e leveduriforme) a caspofungina, nos quais o crescimento da fase leveduriforme foi inibida de 20 a 65 %, enquanto que a fase miceliar de 75 a 82 % (RODRÍGUEZ-BRITO et al., 2010).

1.9. Antifúngicos de origem natural

Uma tendência é o estudo de compostos naturais ou semissintéticos na busca de importantes atividades biológicas, tal como atividade antifúngica. Em 1989 pesquisas envolvendo o composto Ajoene, o qual é um derivado do extrato etanólico de alho (*Alluim sativum L.* família *Liliaceae*), mostrou inibição do crescimento do fungo *P. brasiliensis* afetando a integridade da membrana citoplasmática fúngica (SAN-BLAS et al., 1989; SAN-BLAS et al., 1993; SAN-BLAS et al., 1997). Dois estudos avaliaram o efeito do ajoene em modelo murino, sendo que em um foi apresentado uma redução significativa dos níveis de anticorpos na décima semana de tratamento (MALUF et al., 2008), e em outro mostrou um efeito aditivo positivo quando associado com trimetoprim-sulfametoxazol e protetivo da resposta imune proinflamatória (THOMAZ et al., 2008). Isolados de

Paracoccidioides foram sensíveis a curcumina, um composto produzido pelo rizoma da *Curcuma long*, apresentando efeito inibitório maior do que o agente antifúngico fluconazol (MARTINS et al., 2009). Já dois compostos isolados do extrato de *Schinus terebinthifolius* apresentaram forte atividade anti-*Paracoccidioides,* e um deles quando combinado com itraconazol apresentou efeito sinérgico (JOHANN; CISALPINO*,* et al., 2010; JOHANN; SÁ*,* et al., 2010). O derivado da chalcona, 6-quinolinila foi capaz de controlar a inflamação em modelo murino melhor que o agente itraconazol, evitando a formação de granuloma e preservando o tecido pulmonar (DE SÁ et al., 2015). Em nosso laboratório foram observados que os compostos maitenina e pristimerina oringem destes compostos apresentaram importante atividade contra isolados de *Paracoccidioides* (GULLO et al., 2012), assim como os derivados do ácido gálico que demonstraram uma relação estrutura atividade contra diferentes fungos patogênicos, incluindo as espécies de *Paracoccidioides* (DE PAULA e SILVA et al., 2014).

1.10. Busca de novos fármacos

Para o estudo de novos compostos com potencial atividade antifúngica é preciso lançar mão de um conjunto de técnicas *in vitro* e *in vivo*, para as quais os processos envolvidos não sejam de alto custo e não violem direitos éticos. Experimentos *in vitro* oferecem uma análise básica sobre o alvo de estudo, como por exemplo, a influência direta de um composto sobre o fungo, ou então a influência em um sistema mais complexo. Após obter estas respostas, atualmente a ciência ainda disponibiliza métodos considerados *in vivo* chamados de modelos alternativos, que podem ser utilizados para realizar o pré *screening* dos compostos com atividade promissora, e assim minimizar o uso do modelo murino, os quais são imprescindíveis para uma resposta final, porém com inúmeras restrições para seu uso.

- *Galleria mellonella*

Em 1959, Russell e Burch propuseram a teoria dos três Rs (Reduzir, Reutilizar e Reciclar) almejando tornar o mundo consciente da questão do uso desenfreado de animais na pesquisa, a fim de obrigá-los a buscar alternativas para o uso de modelos animais experimentais (ARORA et al., 2011). A fase larval da *Galleria mellonella* é amplamente utilizada como um modelo animal alternativo (MYLONAKIS; ABALLAY, 2005; FUCHS; MYLONAKIS, 2006; CHAMILOS et al., 2007; MYLONAKIS et al., 2007; PELEG et al., 2009; FUCHS et al., 2010; LIONAKIS, 2011; DESALERMOS et al., 2012). Este modelo

possibilita experimentos com variação de temperatura de 25 a 37 °C, faixa esta que mimetiza as condições do hospedeiro e assim a possibilidade do estudo de infecções fúngicas. Além disso, *G. mellonella* tem seis tipos de células, algumas das quais têm capacidade fagocítica, as quais desempenham um papel importante como sistema de defesa. Alterações na densidade da hemolinfa durante a infecção podem ser facilmente mensuradas e utilizadas como um parâmetro da resposta da larva após a exposição a agentes patogênicos ou substâncias de interesse (FUCHS; MYLONAKIS, 2006; FUCHS et al., 2010; DESALERMOS et al., 2012; JACOBSEN, 2014).

Existem estudos na literatura que correlacionam a eficácia de agentes antifúngicos neste modelo e em testes *in vitro* e *in vivo* (MESA-ARANGO et al., 2013; SCORZONI et al., 2013), como exemplo um estudo que verificou a eficácia de um composto denominado antraquinonas derivado de uma planta *Rubiaceae*, *Morinda tomentosa*, contra o fungo *C. albicans* (FAVRE-GODAL et al., 2014). Neste sentido, preliminarmente o uso de modelos animais alternativos simples tem sido utilizados para gerar os dados preliminares na busca por novos compostos antifúngicos naturais necessários para avançar etapas mais críticas da pesquisa evitando obstáculos da pesquisa antifúngica atual, tais como restrições de custo, tempo e ética. Outras vantagens como temperatura de manutenção similar a utilizada *in vitro* com células de mamíferos e ao hospedeiro, e a semelhança estrutural e funcional da resposta imune inata aos mamíferos, aumentam a credibilidade deste modelo.

1.11. Gênero *Alchornea* e ácido gálico

Por meio de diversos estudos em vários biomas naturais foi possível observar grande ocorrência de espécies do gênero *Alchornea*. Esse gênero tem sido utilizado atualmente como agente antidiarréico, anti-inflamatório, antireumático e no tratamento de hanseníase e de outras doenças cutâneas. A espécie *Alchornea glandulosa* (Euphorbiaceae), é uma árvore de 10-20 metros com caules tortuosos, sendo popularmente conhecida como “tapiá” “tanheiro de folha-redonda”, “tanheiro”, “canela-raposa” ou “amor-seco”, sendo encontrada nas regiões sul e sudoeste do Brasil, e principalmente na Mata Atlântica (PINTO, 2002; CALVO et al., 2007).

Estudos químicos da espécie *A. glandulosa*, revelaram a presença de compostos fenólicos como exemplo o ácido gálico, constituinte majoritário no extrato etanólico. O ácido gálico é um metabólito especial de ocorrência ampla no reino vegetal, o qual apresenta diversas atividades biológicas como anti-inflamatório, antioxidante, antimutagênico, anticarcinogênico, antibacteriana, antiviral, analgésica. Como antioxidante

o ácido gálico poderia atuar como agente antiapoptótico e proteger as células humanas contra danos oxidativos (CALVO et al., 2007; TREVIÑO-CUETO et al., 2007; LOPES et al., 2011).

Devido as suas variadas propriedades e aplicações comerciais, o ácido gálico é um composto de notável interesse para as indústrias farmacêuticas e químicas. Ácido gálico (ácido 3,4,5-triidroxibenzóico) pode ser acumulada na forma livre ou produzida pela hidrólise ácida de ácidos tânicos, porém este possui elevado custo econômico, baixa produção e a pureza como desvantagens (MORAIS et al., 2010; XIMENES et al., 2010; LOPES et al., 2011). Alternativamente, o ácido gálico pode ser produzido por hidrólise microbiana de ácidos tânicos pela tanase (tannin-acylhydrolase EC 3.1.1.20), uma enzima induzível, secretados por microrganismos (POURRAT et al., 1987; INOUE et al., 1995; PINTO, 2002); mas principalmente é uma substância orgânica que ocorre em muitas plantas, como raízes de *Paeonia rockii* (PICERNO et al., 2011), espécies de *Astronium* (DA SILVA et *al.*, 2011), frutos de *Syzygium cumini* (Tobal et al., 2012), *Euphorbia lunulata* (YANG et al., 2011), folhas, caules e raízes de variedades diferentes de *Labisa pumila Benth* (KARIMI et al., 2011), *Zingiber officinale* (GHASEMZADEH ET AL., 2010), caules de Klainedoxa gabonensis (WANSI et al., 2010), *Nervilia aragoana* e folhas de *Atlantia monophylla* (REDDY et al., 2010), *Lawsonia inermis* (SULTANA et al., 2009), *Ardisia chinensis* (LI et al., 2006) e *Alchornea glandulosa* (CALVO et al., 2007).

Há na literatura, trabalhos com ácido gálico e galatos, especialmente estudos focados na relação estrutura atividade destas substâncias. A atividade antimicrobiana de uma série sintética de alcoóis de cadeia longa e aqueles naturalmente encontrados foram estudados contra 15 microrganismos, a fim de adquirir novos conhecimentos sobre suas funções estruturais. Foi observado que a atividade antimicrobiana máxima parece ser dependente do comprimento da cadeia hidrofóbica a partir do grupamento hidroxila (hidrofílico), chamado de efeito *cutoff,* além também de depender do microrganismo a ser testado, ou seja, devem ser consideradas as diferentes morfologias entre os microrganismos testados, como tamanho, forma da célula e espessura de parede (KUBO et al., 1995).

Da mesma forma Kubo et al (2001) avaliaram a atividade antifúngica dos galatos de propila, octila e dodecila, por serem legalmente permitidos para uso como aditivos antioxidantes em produtos alimentícios e cosméticos. Entre os três galatos testados, o galato de octila exibiu atividade antifúngica contra *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii, Candida albicans*, e *Aspergillus niger*. Por isso, o modo de ação fungicida do galato de octila contra *S. cerevisiae* foi avaliada, considerando os

estudos já realizados por Kubo et al, (1995). Assim foi observado que a estrutura cabeça polar e cauda apolar, e ainda a não atividade antimicrobiana específica do galato de octila, foi similar àquela encontrada com os álcoois de cadeia longa em muitos aspectos, e as funções adicionais foram consideradas aos galatos de alquilas. Como no grupo éster não existe uma estrutura álcool, é possível que este grupamento esteja envolvido provocando uma atividade adicional, ou que as esterases presentes no interior da célula hidrolisem o galato de alquila para ácido gálico e os correspondentes álcoois. Concluiu-se que a atividade fungicida do galato de octila depende do comprimento da cadeia alquila e da capacidade de atuar como um agente surfactante não iônico, ou seja, atua como uma substância tensoativa que age como detergente na membrana da celular fúngica (KUBO et al., 2001).

Leal et al (2009) apresentaram resultados da atividade antifúngica de uma série completa de 15 galatos de alquila e seis análogos atuando contra um painel representativo de fungos patogênicos oportunistas. Neste trabalho ressaltaram a importância em considerar o microrganismo testado, a presença de hidroxilas, a influência do comprimento da cadeia e a hidrofobicidade destas substâncias.

Por meio da parceria no Projeto BIOTA-FAPESP e SISBIOTA CNPq, com o Instituto de Química da UNESP de Araraquara, o ácido gálico e seus derivados, denominados galatos de alquila, foram avaliados previamente e classificados como detentores de forte atividade fungicida. Foi observado que a substância ácido gálico apresentou uma CIM (concentração inibitória mínima) que variou de 31,25 a 0,250 mg/L para os isolados das espécies filogenéticas S1, S2 e PS3 de *P. brasiliensis* e para *P. lutzii*, enquanto que os galatos de alquila mostraram valores de CIM entre 16 a 0,004 mg/L, para as mesmas espécies. O menor valor de CIM foi observado para seis galatos que possuem uma substituição por uma cadeia relativamente longa de carbonos, entre este foi destacado o galato de decila (G14). Tais substâncias apresentaram viabilidade celular maior que 80 % nos ensaios de citotoxicidade pelo método de MTT, para duas linhagens celulares pulmonares diferentes (DE PAULA e SILVA et al., 2014). Assim, este trabalho tem como objetivo estudar a influência destas substâncias no sistema *Paracoccidioides*-hospedeiro e compreender o possível mecanismo de ação dos mesmos empregando diferentes metodologias.

2. OBJETIVO GERAL

Este trabalho tem como objetivo correlacionar a interação *Paracoccidioides*-hospedeiro ao mecanismo de ação do galato de decila.

2.1. OBJETIVOS ESPECÍFICOS

I: Analisar a capacidade do G14 em modular a produção de óxido nítrico em macrófagos peritoneais de murino RAW.

II: Analisar as alterações no desenvolvimento de brotamentos e na viabilidade celular pelo ensaio de MTT em *Paracoccidioides lutzii* após contato com o galato de alquila, G14 (galato de decila);

III: Analisar o perfil de interação nos tempos de 1, 3, 6 e 24 horas do fungo *P. lutzii* a:

-linhagens pulmonares humana MRC-5 e A549 pelas metodologias de citometria de fluxo e de imagem;

-linhagem de macrófago alveolar murino AMJ2-C11 pela metodologia de citometria de fluxo;

-hemócitos das larvas de *Galleria mellonella* pela metodologia de citometria de fluxo;

IV: Analisar a capacidade do fungo *P. lutzii* em interagir com as linhagens pulmonares de mamíferos MRC-5 e A549, e em hemócitos de *G. mellonella* por imagens adquiridas no equipamento *In Cell Analyzer*;

V: Analisar a influência do G14 na taxa de interação do fungo *P. lutzii* as linhagens MRC-5 e A549, a linhagem AMJ2-C11 e aos hemócitos por citometria de fluxo*;*

VI: Avaliar a influência do G14 na curva de sobrevivência da larva de *G. mellonella* infectada com *P. lutzii;*

VII: Avaliar o mecanismo de ação dos galatos de alquila por:

-interação químico-genético: analisar o perfil de hipersensibilidade de uma coleção de mutantes conhecidos de *Saccharomyces cerevisiae* frente aos galato de alquila;

-interação genética: comparar o perfil obtido com perfis conhecidos de duplo mutantes em *Saccharomyces cerevisiae*;

VIII: Construir um triplo mutante para bombas de fluxo na levedura selvagem de *Saccharomyces cerevisiae* com genótipo ΔPDR1ΔPDR3ΔSNQ2 pelo método 50:50;

IX: Avaliar em *P. lutzii* e *P. brasiliensis* após contato com G14 a taxa de atividade de enzimas n-glicosiladas ligadas à formação da parede celular e a expressão relativa dos genes envolvidos através de PCR em tempo real.

3. MATERIAIS E MÉTODOS

3.1. Composto com atividade anti-*Paracoccidioides*

Por meio da parceria no Projeto BIOTA-FAPESP e SISBIOTA CNPq, com o Instituto de Química da UNESP de Araraquara. Inicialmente o ácido gálico (Sigma) obtido comercialmente e, posteriormente, foi submetido a modificações por reações de esterificação e então foram obtidas diferentes moléculas com tamanhos de cadeia carbônicas diferentes, chamados galatos de alquila. O ácido gálico e os galatos de alquila foram avaliados previamente por De Paula e Silva (2014) e classificados como detentores de forte atividade fungicida. Entre estes foi destacado o galato de decila (G14) com excelente atividade antifúngica contra o genêro *Paracoccidioides*.

3.2. Fungo gênero *Paracoccidioides*

3.2.1. Meios de cultura

a. Meio Fava-Netto

Este meio foi preparado semanalmente em água destilada com os seguintes componentes: 0,3% de proteose peptona, 1 % de Peptona A, 0,5 % de extrato de carne, 0,5 % de extrato de levedura. 0,5 % de cloreto de sódio, 4 % de dextrose e 2 % de ágar bacteriológico. O meio foi distribuído em tubo de ensaio de vidro devidamente selados com tampões de algodão e esterilizados em autoclave. Foram mantidos a 4 °C por uma semana.

b. Meio BHI (*Brain heart infusion*)

Este meio foi adquirido comercialmente e foram preparados 100 mL em água destilada, acrescido de 1 % de glicose, em Erlenmeyer de capacidade de 500 mL e esterilizados em autoclave. Foi mantido a 4 °C por uma semana.

c. Meio YEP (*Yeast extract peptone*)

Este meio foi preparado em água destilada com os seguintes componentes: 2 % de peptona A e 1 % de extrato de levedura. Foi esterilizado em autoclave e mantido a 4 °C por uma semana.

3.2.2. Condições gerais

O fungo *P. lutzii* (ATCC MYA-826) (Pl01) e *P. brasiliensis* isolado 18 (Pb18) são procedentes da micoteca da Faculdade de Medicina da Universidade de São Paulo (FM-USP). Durante a realização deste trabalho, ambos foram mantidos em meio de Fava-Netto em estufa a 37ºC. Para a manutenção, foram realizados repiques semanalmente até o décimo repique. Para os experimentos foram utilizados repiques com 4 ou 5 dias. A virulência do fungo foi reativada por inoculação em camundongo e o primeiro repique foi mantido em câmara fria.

3.2.3. Inóculo

O fungo foi cultivado de 4 a 5 dias, a 37 °C, em meio de Fava-Netto sólido. O crescimento foi desprendido do meio com auxílio de uma alça e transferido para um Erlenmeyer com 100 mL de meio BHI suplementado. A suspensão foi incubada a 150 RPM a 37 °C por 3 dias. A suspensão foi centrifugada a 5000 RPM por 5 minutos e o pellet ressuspendido em PBS. A suspensão foi passada várias vezes por uma agulha 1,20 x 40 mm (BD) e então filtrada no filtro de nylon de 10 µm (BD Falcon). O inóculo foi disposto em câmara de Neubauer, e a concentração de células fúngicas foi estimada de acordo com o percentual de células viáveis, verificado pelo método de azul de tripano.

3.3. Modelo Cultura celular

3.3.1. Meio de cultura

O meio DMEM (*Dulbecco's Modified Eagle Medium-Sigma*) foi adquirido comercialmente e suplementado com 0,15 % de bicarbonato de sódio. Para as linhagens de macrófagos e a linhagem A549 foi também usado 0,35 % de glicose. O meio foi

esterilizado por sistema de filtração descartável com diâmetro do poro de membrana de 0,22 μm. O meio foi conservado a 4 °C, e para o uso foi uma alíquota foi em temperatura ambiente foi suplementada com 10% de SFB (soro fetal bovino) e 1 % de solução de antibiótico (Sigma).

3.3.2. Condições gerais

Todos os tipos celulares foram mantidos a uma temperatura de 37°C sob 5 % CO_2 em garrafas para cultura de tecidos 75 cm^2, por 3 a 4 dias, até a formação da monocamada com confluência de 100 %. As células foram recolhidas da garrafa (de acordo com o tipo celular). Para uma placa de 96 poços, cálculos foram realizados para concentração final de 10^5 células por poço, de acordo o percentual de células viáveis, verificado pelo método de azul de tripano. A placa foi incubada a 37°C sob 5 % CO_2, de 18 a 24 horas para a adesão e formação da monocamada com confluência de 100 %.

a. Linhagens celulares murinas

As linhagens de macrófago alveolar de camundongo (AMJ2-C11) (Banco de Células do Rio de Janeiro) e peritoneal de camundongo (RAW) foram cultivadas em meio DMEM suplementado com adição de glicose. Para recuperar as células aderidas no aparato da garrafa foi utilizado o *cell scrapper*.

b. Linhagens celulares humanas

A linhagem pulmonar humana A549 (epitelial-carcinoma) foi cultivada em meio DMEM suplementado com adição de glicose. A linhagem pulmonar humana MRC-5 (fibroblasto – normal) foi cultivada em meio DMEM suplementado sem adição de glicose. Ambas foram obtidas do Banco de Células do Rio de Janeiro. A técnica da tripsina foi utilizada para recuperar as células aderidas no aparato da garrafa.

3.4. Modelo *Galleria mellonella*

3.4.1. Condições gerais

As larvas foram mantidas em cera de abelha e pólen a 25°C (RAMARAO et al., 2012) até atingirem o peso entre 150-200 mg. Larvas com coloração clara, sem manchas e na faixa de peso adequado foram separadas em placas de Petri e incubadas a 37°C por 16 horas com proteção da luz. Antes de cada ensaio, as regiões das pro-patas foram limpas com álcool 70%. Foram reservadas 8 larvas para cada grupo em placas de Petri para os

ensaios de toxicidade do composto, curva de sobrevivência, ensaio de interação e ensaio de interação com tratamento.

3.5. Ensaio MTT

O ensaio de MTT é avalia de forma quantitativa a sobrevivência e proliferação celular de forma indireta, através da determinação da atividade mitocondrial. Este ensaio é considerado um método colorimétrico que é determinado pela redução do sal tetrazólico MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio) em um precipitado formado pela ação da enzima succinato desidrogenase, produto da respiração mitocondrial, chamado formazana. Esta conversão ocorre somente em células vivas e a quantidade do precipitado é proporcional ao número de células vivas. O precipitado de formazana apresenta coloração azul e com a adição de um solvente orgânico, como o DMSO (dimetilsulfóxido), é possível sua solubilização (DENIZOT; LANG, 1986; GERLIER; THOMASSET, 1986; FERRARI et al., 1990).

O ensaio de MTT foi realizado para as células fúngicas de *P. lutzii* e para as células de mamíferos (linhagens de macrófagos murino e linhagens pulmonares humanas). Para todos os ensaios, como controle foram utilizados poços somente com o meio (branco), poços com células sem tratamento (somente com quantidade proporcional de DMSO utilizado para diluir o composto - controle positivo) e poços com células sem tratamento com 50 % de DMSO (controle de morte). Estes controles foram utilizados para ter como parâmetro a atividade metabólica máxima e mínima em cada tempo. Após 24 horas de contato do composto com as células, o sobrenadante foi retirado cuidadosamente e uma alíquota de 10 µL de solução de MTT (5 mg/mL em PBS) e mais 40 µL de PBS foram adicionados por poço. A placa foi incubada a 37°C sob 5 % CO_2 por no mínimo 4 horas. Após o tempo de incubação, foram adicionados 100 µL de DMSO puro por poço para a solubilização do precipitado de formazana e a placa foi submetida a leitura por espectrofotômetro a 570 nm (690 nm referência).

3.6. Quantificação da concentração de nitritos no sobrenadante da cultura de macrófago RAW após contato com o composto G14.

Este ensaio foi realizado no laboratório de Imunologia da Professora Dra Alexandra Ivo Medeiros, da Faculdade de Ciências Farmacêuticas de Araraquara - UNESP, com auxílio do Dr. Gustavo Nazzi.

Um método para a determinação indireta de NO envolve a análise espectrométrica pelo ensaio de Griess, que quantifica os nitritos acumulados no sobrenadante de culturas celulares, uma vez que o NO origina nitritos em solução aquosa. O ensaio de Griess envolve uma reação de diazotização de dois passos, no qual um derivado intermediário de NO reage com a sulfanilamida produzindo um sal de diazônio o qual se liga ao NEED (*N-1-naphthyl-ethylenediamine-dichloroplatinum*) formando um produto rosa que possui espectro de absorção característico e que pode ser quantificado por espectrofotometria.

Após 18 horas de incubação da placa para a formação da monocamada de macrófagos peritoneais de murino RAW, o sobrenadante foi retirado e 100 µL de cada concentração de G14 (0,13 a 1 µg/mL) em DMEM suplementado e com adição de glicose foram dispostos sob a monocamada. Cada concentração foi realizada em triplicata. Como controles foram utilizados três poços com 100 µL de meio DMEM suplementado e com adição de glicose sob a monocamada celular (controle sem estímulo) e outros três poços com 100 µL de meio DMEM suplementado e com adição de glicose, acrescido de 20 µL de LPS (lipossacarídeo) a 1 mg/mL sob a monocamada celular (controle com estímulo). As placas foram incubadas a 37 °C sob 5 % CO_2, por 24 horas.

O mesmo esquema foi seguido, porém com a presença de LPS, assim foram adicionados 20 µL por poço de LPS a 1 mg/mL. Após o tempo de 24 horas de incubação, o sobrenadante de ambas as situações (monocamada tratada com G14 e estimulada por LPS, e monocamada tratada com G14 e não estimulada por LPS) foram transferidos para outra placa e uma alíquota 100 µL da solução fresca de NEED a 0,1 % e sulfanilamida a 1 % (v/v - 1:1) foi adicionado por poço. A leitura em espectrofotômetro foi realizada a 540 nm. A concentração de nitrito na amostra foi calculada de acordo com uma curva de uma concentração inicial de 200 µM em meio DMEM. A placa com a monocamada de macrófago restante foi conduzida para ensaio de MTT, de acordo com o item 3.5., no intuito de monitorar a viabilidade celular durante o teste. Foram realizados três experimentos independentes. Gráficos e análises estatísticas foram realizadas com Graph Pad Prisma 5 (La Jolla CA, USA), utilizando ANOVA com Tukey's post-test.

3.7. Análise do composto G14 na atividade mitocondrial e no desenvolvimento das células fúngicas de *P. lutzii*.

Em ensaios realizados anteriormente, foi observado que $2{,}5.10^2$ células fúngicas de *P. lutzii* (Pl01) por poço foram inibidas com 0,13 µg/mL de G14 (DE PAULA e SILVA et al., 2014). Como será demonstrado posteriormente, nos ensaios de interação foram

utilizadas 10^5 células fúngicas por poço. Nesse sentido, foram testadas diferentes concentrações de do composto G14 para encontrar a melhor CIM para 10^5 células fúngicas por poço.

Para este ensaio um inóculo foi preparado como descrito no item 3.2.3. Em uma placa de 96 poços, 10^5 células fúngicas por poço em meio BHI caldo acrescido de 1 % de glicose foram desafiadas com as concentrações finais de 0,5 a 0,13 μg/mL do composto G14. Cada concentração foi realizada em quadruplicata. A placa foi incubada a 37°C sob 5 % CO_2 por 24 e 48 horas. Como controle positivo foram utilizados poços com células fúngicas somente com meio BHI caldo acrescido de 1 % de glicose e sem o composto G14, para o tempo inicial (T0), de 24 (T24) e de 48 (T48) horas de contato. Em cada tempo, uma alíquota de 10 μL de cada poço, de cada concentração, foi submetida em câmara de Neubauer para análise. Foram contabilizadas 100 células fúngicas, entre as quais foram contadas aquelas que não apresentavam e que apresentavam brotamento, levando uma relação da taxa de células com brotamento. Ao final da análise da formação de novos brotamentos de cada tempo, o ensaio de MTT foi conduzido de acordo com o item 3.5. Foram realizados três experimentos independentes. Análises estatísticas foram realizadas com Graph Pad Prisma 5 (La Jolla CA, USA), utilizando ANOVA com Tukey's post-test.

3.8. Protocolos de marcação

Os ensaios de interação das células fúngicas de *P. lutzii,* foram realizados com a linhagem de macrófago alveolar murino AMJ2-C11, com as linhagens pulmonares humanas A549 e MRS-5, e com os hemócitos de *G. mellonella*. As interações foram analisadas por citometria de fluxo e/ou citometria de imagem, nas quais as populações foram distinguidas principalmente por fluoróforos.

3.8.1. Marcação das células fúngicas

Uma suspensão de 5.10^8 células fúngicas/mL foi marcada com o fluorocromo 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE/Sigma Aldrich) na concentração final de 15 μM. CFDA-SE, devido ao grupo acetato, é altamente permeável na célula e é uma molécula não fluorescente, assim esterases presentes no citoplasma da célula removem o grupo acetato convertendo a um éster fluorescente (ex/em 492/517). O grupo succinimidil se liga covalentemente aos resíduos de lisina e a outras fontes de amina intracelular, permanecendo durante longos períodos dentro das células e assim não é transferido para células adjacentes. Esta suspensão foi incubada por 20 minutos a 37 °C e

lavada por três vezes com PBS (5000 RPM, 5 minutos). O pellet foi ressuspendido em PBS para o volume inicial. Células sem marcação foram utilizadas como controle. Os dados foram analisados em gráficos de histograma. A partir do controle sem marcação, foi criado um gate para as células fúngicas marcadas com CFDA-SE. Uma parte de cada amostra analisada foi submetida ao teste de viabilidade por alamar Blue.

3.8.2. Marcação das células de mamífero e hemócito

Uma solução a 0,165 μM de Alexa Fluor® 647 *phalloin conjugates* (Invitrogen) em 4% de paraformaldeído gelado (Sigma) acrescido de 0,5% de Triton X-100 (solução de fixação-permeabilização - Amersham Biosciences) foi preparada no momento do ensaio. Para cada 10 μL de hemolinfa de cada larva ou a cada poço com a monocamada de célula de mamífero foram utilizados 50 μL desta solução. Para cada tempo foi conduzido um controle marcação positiva (somente monocamada celular ou hemolinfa acrescida da solução de marcação faloidina) e outro controle negativo (somente com a solução fixação-permeabilização sob a monocamada celular ou hemolinfa, sem marcação). Faloidina é um peptídeo bicíclico pertencente a uma família de toxinas isoladas do cogumelo *Amanita phalloides* e possui seletividade pelos filamentos de actina. Faloidina ligada a Alexa Fluor 647 fornece alto contraste para a discriminação da actina e fotoestabilidade em excitação/emissão 650/668 nm.

3.9. Parâmetros utilizados para análise por citometria de fluxo

As interações foram adquiridas no equipamento BD FACS Canto, e analisadas pelo programa BD FACS Diva software. Um gráfico *dot plot* SSC (*Side SCatter*) versus FSC (*Forward SCatter)* foi construído para determinar as diferentes populações (células de mamífero/hemócitos da larva de *G. mellonella*/células fúngicas). A população de células de mamífero/hemócitos da larva de *G. mellonella* foi delimitada pelo gate P1, do qual foi derivado o gráfico *dot plot* SSC versus APC (filtro para a fluorescência Alexa Fluor 647) para distinguir as células Alexa Fluor 647 positivas, gerando o gate P2. Um gráfico *dot plot* SSC vesus FITC (filtro para a fluorescência CFDA-SE) foi derivado do gate P2 para analisar o número de células CFDA-SE positivas dentro desta população Alexa Fluor 647 positivas gerando o gate P3 (Figura 1).

Desse modo, obteve-se a taxa de células de mamífero/hemócitos da larva de *G. mellonella* positivas para Alexa Fluor 647 interagindo com células fúngicas positivas para CFDA-SE. Nesta análise de interação não é possível determinar se a célula fúngica

encontra-se aderida ou se houve internalização na célula de mamífero ou hemócito, portanto a dupla marcação foi considerada como interação. A situação sem marcação foi utilizada como controle negativo. Histogramas foram adquiridos para a determinação destes parâmetros. Todas as amostras analisadas foram diluídas em solução FACS e foram adquiridos 10000 eventos dentro do gate P2 (Figura 1) no modo de aquisição médio. Neste modo, a relação da porcentagem de interação foi obtida. Gráficos e análises estatísticas foram realizadas com Graph Pad Prisma 5 (La Jolla CA, USA), utilizando ANOVA com Tukey's post-test.

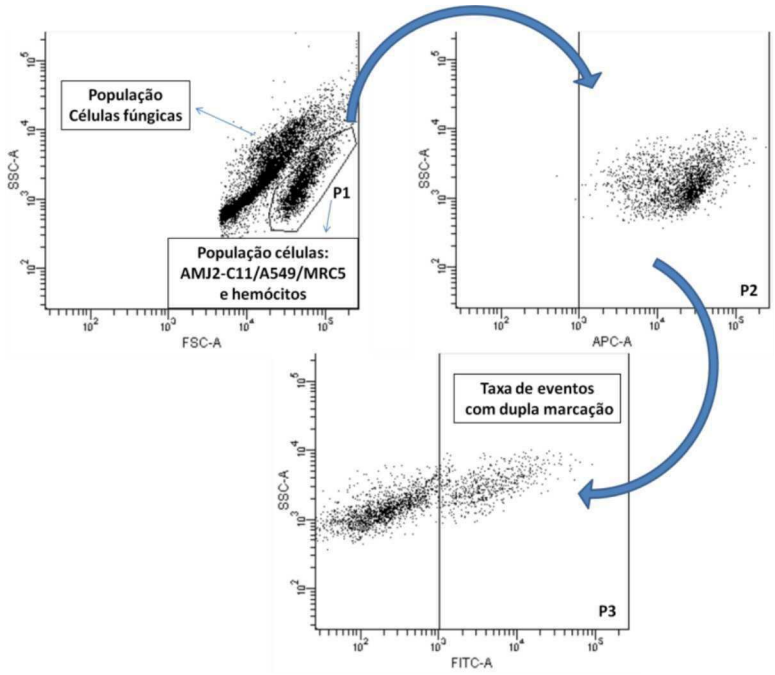


Figura 1 - Esquema para análise por citometria de fluxo no ensaio de interação com dupla marcação. Equipamento: BD FACS Canto. Software de análise: BD FACS Diva. P1: *gate* população de células de mamífero (macrófagos, linhagens pulmonares) e hemócitos da larva *G. mellonella*. P2: *gate* derivado da população P1, população positiva para a marcação faloidina. P3: *gate* derivado da população P2, positiva para CFDA-SE.

3.10. Parâmetros utilizados para análise por citometria de imagem

As imagens dos poços foram adquiridas utilizando o equipamento *In Cell Analyzer* 2000 – GE. Foram adquiridos dois poços por tempo com 30 campos cada, utilizando a objetiva de 20X. O núcleo marcado com DAPI (*4',6-Diamidino-2-Phenylindole*) foi detectado pelo filtro *brightfield*-DAPI, as células fúngicas de *P. lutzii* marcadas com CFDA-SE foram detectadas no filtro FITC e as células de mamífero das linhagens pulmonares MRC-5 e A549, e os hemócitos da larva de *G. mellonella* marcados com faloidina - Alexa Fluor 647 foram detectados no filtro *Cy5*. Na análise das imagens

adquiridas pelo software *In Cell Investigator* foi utilizado o modo *multi target analysis*, com área mínima de 50 μm^2 e sensibilidade de 100. O núcleo foi identificado no *wave* 1 (*top-hat*); para a detecção das células fúngicas, estas foram categorizadas como organela no *wave* 2 (*multiscale top-hat*) devido a limitação do programa; e as células de mamífero das linhagens pulmonares MRC-5 e A549, e os hemócitos da larva de *G. mellonella* foram detectadas no *wave* 3 (*region growing*). Dois grupos foram determinados: interação e não interação. Como critério para a o grupo interação célula/fungo foi determinado a distância mínima de 10 µm do centro do núcleo celular até o centro da célula fúngica (figura 2).

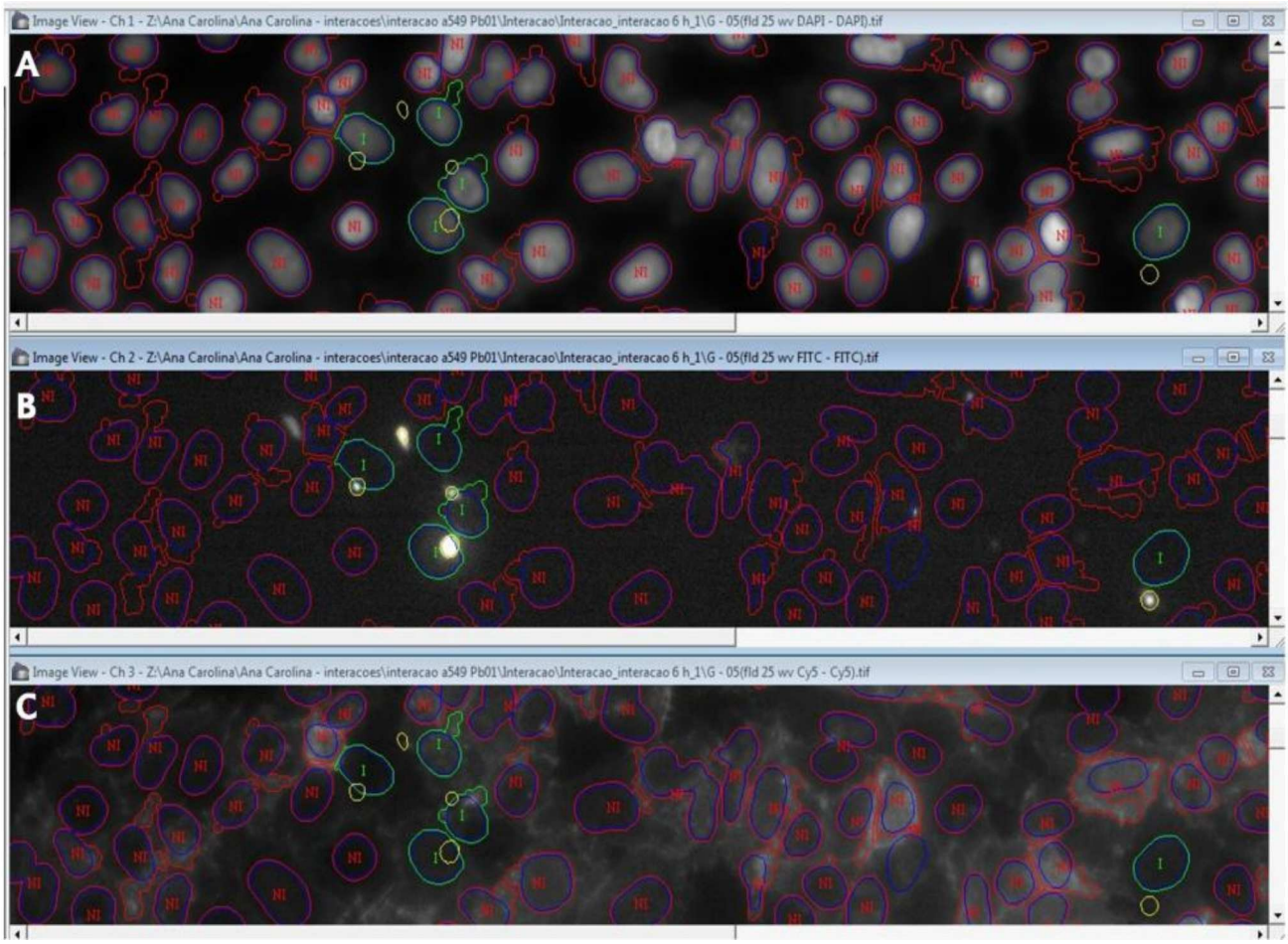


Figura 2 - Esquema para análise por citometria de imagem no ensaio de interação com dupla marcação. Equipamento: *In Cell Analyzer 2000*. Software de análise: *In Cell Investigator*. As células foram detectadas de acordo com o marcador fluorescente e os parâmetros de tamanho estabelecidos. (A): *wave* 1 – núcleo/DAPI, (B): *wave2:* fungo/ CFDA-SE, (B) *wave* 3: células/ faloidina - Alexa Fluor 647. Circulo azul: detecção da célula; circulo amarelo: detecção do fungo; circulo vermelho (NI): não detecção de interação; circulo verde (I): detecção de interação.

3.11. Ensaio de interação célula de mamífero e células fúngicas de *P. lutzii*

Nesta etapa, os ensaios de interação realizados com a linhagem de macrófago alveolar de murino AMJ2-C11 foram realizados no laboratório de Imunologia da Professora Dra Alexandra Ivo Medeiros, da Faculdade de Ciências Farmacêuticas de Araraquara - UNESP, com auxílio da Doutoranda Ana Carolina Salina. As análises e os ensaios de interação com as linhagens pulmonares humanas MRC-5 e A549 foram realizadas em nosso laboratório.

3.11.1. Citometria de imagem

Após a formação da monocamada celular das linhagens pulmonares humanas MRC-5 e A549, o sobrenadante foi retirado cuidadosamente. A cada poço foram adicionados 100 µL do inóculo com 10^5 células fúngicas marcadas com CFDA-SE em meio específico. A placa foi incubada por 1, 3, 6 e 24 horas a 37°C sob 5 % CO_2. Após o período de incubação, o sobrenadante foi retirado, os poços foram lavados 3 vezes cuidadosamente com PBS e 50 µL da solução de marcação faloidina foram adicionados nos poços por 20 minutos. A placa foi conduzida para análise no aparelho *In Cell Analyzer* 2000 – GE

3.11.2. Citometria de fluxo

Após a formação da monocamada celular das linhagens pulmonares humana MRC-5 e A549, e da linhagem de macrófago alveolar de murino AMJ2-C11, o sobrenadante foi retirado cuidadosamente. A cada poço foram adicionados 100 µL do inóculo com 10^5 células fúngicas marcadas com CFDA-SE em meio específico. A placa foi incubada por 1, 3, 6 e 24 horas a 37°C sob 5 % CO_2. Após o período de incubação, o sobrenadante foi retirado, a interação foi lavada 3 vezes cuidadosamente com PBS e 50 µL da solução de marcação faloidina foram adicionados nos poços por 20 minutos. Para a linhagem de macrófagos AMJ2-C11, 200 µL de PBS gelado foram adicionados por poço para desprendê-las do aparato da placa (BERNARDINO et al., 2013). As linhagens celulares A549 e MRC-5 foram desprendidas do aparato da placa pelo método de tripsina. A suspensão de cada poço foi ressuspendida em solução FACS e submetida à análise por citometria de fluxo, no aparelho BD FACS Canto.

3.12. Ensaio de interação hemócito e células fúngicas de *P. lutzii*

Primeiramente os ensaios foram conduzidos com 5.10^6 células fúngicas por larva, como estabelecido em nosso laboratório (SCORZONI et al., 2015). Porém, a partir de 3 horas de infecção, a larva apresentou drástica perda de volume da hemolinfa, o que inviabilizou a análise em 24 horas como padronizado. Neste sentido, foi utilizada para os experimentos a concentração de 10^6 células por larva garantindo que as larvas não apresentassem um estado de debilidade crítica e a análise pudesse ser realizada com mais precisão.

3.12.1. Citometria de fluxo

Com o auxílio de uma seringa (Hamilton, USA), 10 µL com 10^6 células fúngicas, previamente marcadas com CFDA-SE, foram injetadas na última pro-pata esquerda da larva (THOMAZ et al., 2013). Estas foram incubadas a 37 °C por 1, 3, 6 e 24 horas. Para cada situação foram utilizadas 5 larvas em 3 experimentos independentes. Larvas foram inoculadas com PBS como controle. Após o tempo de incubação uma pequena incisão na lateral baixa do corpo da larva da *G. mellonella* foi realizada, e 10 µL de hemolinfa foram retirados e colocados em um poço de uma placa de 96 poços contendo 50 µL da solução de marcação faloidina e incubados por 20 minutos a 4°C, protegido da luz. Cada amostra foi resuspendida em 200 µL de solução FACS e submetidos análise por citometria de fluxo, no aparelho BD FACS Canto.

3.13. Aquisição de imagens *In Cell Analyzer*

As imagens adquiridas no aparelho *In Cell Analyzer* 2000 – GE para a análise por citometria de imagem foram obtidas no tempo de 6 horas da interação fungo e células pulmonares (A549 e MRC-5), posteriormente analisadas e selecionadas para demonstrar a capacidade de interação Pl01 e célula. Para a interação hemócito/fungo no tempo de 6 horas uma parte foi processada, que seria analisada por citometria de fluxo, e outra foi encaminhada para a aquisição de imagens no aparelho *In Cell Analyzer* 2000 – GE. Em ambas as interações foram selecionadas imagens para elucidar a marcação das células pela faloidina-Alexa648 e do fungo por CFDA-SE, além de comprovar a capacidade do fungo em aderir ou internalizar-se na célula.

3.14. Citotoxicidade em cultura celular

O teste de citotoxicidade foi conduzido de acordo com o tempo total utilizado para o ensaio de interação. Por isso, após a formação da monocamada, o sobrenadante foi retirado cuidadosamente e 100 µL de meio específico foram adicionados por poço. A placa foi incubada por mais 6 horas a 37°C sob 5 % CO_2 devido ao tempo total de incubação do ensaio de interação que será descrita posteriormente. Após 6 horas, o sobrenadante foi retirado e 100 µL de cada concentração do G14, diluído em meio específico para cada tipo celular, foram dispostos nos poços com a monocamada. A placa foi incubada por 24 horas a 37°C sob 5 % CO_2. Após o tempo de tratamento o ensaio de MTT foi conduzido, como descrito no item 3.5. Foram realizadas quadruplicatas em três experimentos independentes. Análises foram realizadas com Graph Pad Prisma 5 (La Jolla CA, USA), utilizando ANOVA com Tukey's post-test.

3.15. Análise da influência do G14 na taxa de interação por citometria de fluxo

3.15.1. Interação célula de mamífero e células fúngicas de *P. lutzii* desafiada com o composto G14

Após a formação da monocamada celular das linhagens pulmonares humana MRC-5 e A549, e da linhagem de macrófago alveolar de murino AMJ2-C11, o sobrenadante foi retirado cuidadosamente. A cada poço foram adicionados 100 µL do inóculo com 10^5 células fúngicas marcadas com CFDA-SE em meio específico para cada tipo celular. A placa foi incubada por 6 horas a 37°C sob 5 % CO_2. Após o período de incubação, o sobrenadante foi retirado e foram adicionados 100 µL do composto G14, diluído em meio específico para cada tipo celular na concentração de 0,5 µg/mL. A placa foi incubada por 24 horas a 37°C sob 5 % CO_2. Após o período de incubação, o sobrenadante foi retirado, a cada poço foi lavado 3 vezes cuidadosamente com PBS e 50 µL da solução de marcação faloidina foram adicionados nos poços por 20 minutos. Para os macrófagos, um volume de 200 µL de PBS gelado foi adicionado por poço (BERNARDINO et al., 2013). As células A549 e MRC-5 foram desprendidas do aparato da placa pelo método de tripsina. A placa foi incubada a 37°C por 5 minutos, as células foram resuspendidas em solução FACS e submetida análise por citometria de fluxo.

3.15.2. Interação hemócitos e células fúngicas de *P. lutzii* desafiada com o composto G14

Com o auxilio de uma seringa (Hamilton, USA), 10 µL com 10^6 células fúngicas, previamente marcadas com CFDA-SE, foram injetadas na última pro-pata esquerda da larva (THOMAZ ET AL., 2013). Estas foram incubadas a 37 °C por 6 horas. Para cada situação foi utilizado um n de 5 larvas em 3 experimentos independentes. Larvas foram inoculadas com PBS como controle. O composto G14 foi diluído em PBS na dose equivalente a concentração de 0,5 µg/mL. Após o tempo de 6 horas de infecção, cada larva foi tratada com G14 inoculando 10 µL na pró-pata e incubadas a 37°C por 24 horas. Após o tempo de incubação uma pequena incisão na lateral baixa do corpo da larva da *G. mellonella* foi realizada, e 10 µL de hemolinfa foram retirados e colocados em um poço de uma placa de 96 poços contendo 50 µL da solução da solução de marcação faloidina e incubados por 20 minutos a 4°C, protegido da luz. A suspensão de cada poço foi ressuspendida em solução FACS e submetida à análise por citometria de fluxo, no aparelho BD FACS Canto.

3.16. Curva de sobrevivência da larva *G. mellonella* frente ao G14.

Com o auxilio de uma seringa (Hamilton, USA), 10 µL com 10^6 células fúngicas foram injetadas na última pro-pata esquerda da larva (THOMAZ et al., 2013). Estas foram incubadas a 37 °C por 6 horas. Larvas foram inoculadas com PBS como controle. Após o tempo de incubação, cada larva foi inoculada com 10 µL do composto G14, diluído em PBS na dose equivalente a concentração de 0,5 µg/mL, na pró-pata com auxílio da seringa Hamilton e incubadas a 37°C por 10 dias. Um grupo controle somente com PBS e outro tratado com o composto G14, ambos sem infecção, foram conduzidos. As larvas do grupo controle que receberam o tratamento e morreram representaram a toxicidade do composto. Para avaliar a efetividade do composto, a curva de sobrevivência do grupo infectado foi comparada com o grupo infectado e tratado. Para a determinação de morte foi considerado a falta de movimento após tocá-las com uma pinça. Para cada situação foram utilizadas 16 larvas em três experimentos independentes.

3.17. Avaliação do mecanismo de ação dos galatos de alquila

Esta fase do trabalho foi realizada na Universidade de Toronto, no laboratório "*the Donnelly Centre for Cellular + Biomolecular Research; Banting ans Best Department of*

Medical Research", sob a orientação do Professor Dr. Charles Boone, no período de agosto de 2014 a janeiro de 2015.

3.17.1. Ensaio de interação químico-genético

É uma poderosa ferramenta para entender o mecanismo de ação e os alvos de compostos desconhecidos, de um modo prático e rápido, contribuindo na geração de uma biblioteca de informações, as quais podem ser analisadas sob o ponto de vista tanto do hospedeiro quanto do patógeno (PARSONS et al., 2004; PARSONS et al., 2006; HO et al., 2011; WILLIAMS et al., 2011; FUNG et al., 2014). Os principais pontos fortes desta metodologia relacionam-se ao ensaio que envolve a célula como um todo, e não somente um alvo específico, além de promover uma visão imparcial da resposta celular ao composto (FUNG et al., 2014). A técnica é baseada na exposição de uma grande coleção de mutantes por deleção de genes específicos a compostos e avalia-se o mutante individualmente (GIAEVER et al., 2004), uma vez que a condição individual de cada mutante pode ser rastreada por sequenciamento já que os mutantes específicos carregam identificações moleculares (SMITH et al., 2009).

3.17.1.1. Modelo *Saccharomyces cereviseae*

A partir da coleção *Yeast Knochout collection - Yeast MAT a Collection* (GE HealthCare Dharmacon), a coleção chamada *Mini-Pool* foi elaborada pelo laboratório do Professor Charlie Boone (Universidade de Toronto, Toronto, Canadá) que consiste em uma coleção reduzida com 310 cepas mutantes para genes funcionalmente representativos no genoma geral da levedura. Alíquotas de 200 µL em uma concentração final de 250 células por cepa a cada 2 µL em YEP (*yeast extract and peptone)* acrescido de 2 % de glicose adicionado de Geneticin418 (200 mg/mL G418), e glicerol 15 % foram mantidas a -80 ^{o}C até o momento do uso.

3.17.1.2. Teste de sensibilidade

No ensaio para a avaliação do perfil Químico-Genético, em uma placa de 96 poços foram adicionados 196 µL de YEP acrescido 2 % de galactose, 2 µL do inóculo previamente ajustado para 250 células por cepa e 2 µL de cada composto em diferentes concentrações (ácido gálico e os galatos de: hexila, heptila, nonila, decila, undecila, dodecila e tetradecila). Foram utilizados como controle os fármacos Tunicamicina (inibidor glicosilação de proteínas), MMS (agente indutor de danos ao DNA), Micafungina

(antifúngico), Bortezomib (inibidor de proteassoma), Benomyl (agente despolimerizante de microtúbulo) e o DMSO puro como controle do solubilizante (quantidade referente a utilizada com os compostos). Na primeira avaliação, os compostos foram testados na faixa de concentração 100 a 0,75 µM. A placa foi incubada por 24 horas a 30 °C e então a DO foi adquirida a 538 nm. Os poços que apresentaram crescimento entre 50-80 % em relação ao crescimento do controle DMSO, foram selecionados. Para um estudo mais preciso, uma nova avaliação foi realizada utilizando concentrações intermediárias às selecionadas. Determinada a concentração que apresentou crescimento entre 50-80 %, o poço selecionado foi coletado e centrifugado (10000 RPM, 2 minutos). Os controles também foram processados.

3.17.1.3. Extração DNA genômico

O pellet obtido foi submetido à extração do DNA genômico pelo kit *Master Pure Yeast DNA Purification* (Epicenter).

3.17.1.4. Sequenciamento plataforma *Illumina*

3.17.1.4.1. *PCR*

Cada mutante possui uma deleção com uma marca (*barcode*) desenhada e otimizada para plataforma *Illumina* visando seu sequenciamento. Aos DNAs genômicos dos mutantes em cada condição (concentração do composto selecionado), foi adicionado um iniciador único de 10 pb, também desenhado e otimizado para a plataforma *Illumina* na concentração de 12,5 M, o que permitiu a mistura posteriormente de todas as condições em um único sequenciamento. A cada *barcode* há agregada uma sequencia comum a todas as deleções. Assim foi utilizado 2,5 µL (1µM) do *reverse primer,* denominado *common* U2 (SCL_Barseq–Invitrogen-CAAGCAGAAGACGCATACGAGCTCTTCCGATCTGCACGTCAAGACTGTCAAGG), 16 µL de *Super Mix solution* (Platinum PCR – Invitrogen) e 2,5 µL da extração do DNA genômico. As condições da PCR foram: 5 minutos a 95 ^{o}C (inicial desnaturação), seguido de 30 ciclos de 1 minuto a 95 ^{o}C (desnaturação), 30 segundos a 55 ^{o}C (anelação), 45 segundos a 68 ^{o}C (extensão). A extensão final foi realizada com 10 minutos a 68 ^{o}C. Um volume de 5 µL do produto de PCR foi adicionado a 2 µL *6xOrange DNA loading Dye* (Thermo Scientific) e 5 µL água destilada estéril, e submetido a eletroforese em gel de agarose 2 % por 30 minutos a 120 volts. Produto do tamanho de 267 pb.

3.17.1.4.2. Processamento da amostra

Após a confirmação da integração da marcação a todas as condições selecionadas, 10 µL de cada produto de PCR obtido foi misturado em um único microtubo e quantidade proporcional de 6x *Orange DNA loading Dye* (Thermo Scientific) foi adicionado. A amostra total foi submetida a eletroforese em gel de agarose 2 % por 30 minutos, a 120 volts. A partir do gel foi feita uma extração do produto com 267 pb utilizando o kit *QIA Quick Gel Extraction* (Qiagen) adquirindo assim a biblioteca de DNA. As bibliotecas foram quantificadas pelo kit *Library Quantification* (*Illumina Sequencing Plataforms* – KAPA) por PCR em tempo real pelo *LightCycler* 480 qPCR. As amostras foram enviadas para sequenciamento utilizando o kit *MiniSeq Reagent* v3 (*Illumina*). Através do sequenciamento foram obtidas informações quantitativas do número de cópias gênicas de cada mutante presente em cada situação, normalizadas de acordo com o controle DMSO.

3.17.2. Interação genética

O perfil da interação químico-genético da coleção de mutantes *Minipool* foi posteriormente pareado com uma rede de interações genéticas da levedura, a qual é constituída por um banco de dados de duplo mutantes, para predizer a via e as proteínas que os compostos estão possivelmente afetando. Esta metodologia pode ser usada em vários tipos de biomoléculas (LOPEZ et al., 2008; COSTANZO et al., 2010).

Com a vantagem de criação de um banco de dados que representa a grande maioria dos genes de *Saccharomyces cerevisiae* anotado, o desempenho do mutante na presença do composto pode levar a um suposto mecanismo de ação. Por exemplo, na presença de agentes que danificam o DNA como MMS (metil metanosulfonato), os mutantes gerados que envolvem as deleções na via de reparo aos danos no DNA (ex. Δrad51) são significativamente mais sensíveis para o agente do que os outros mutantes. A coleção de mutantes pode render um valioso conhecimento para compostos com bioatividade fraca, e a sensibilidade do mutante pode ser muito alta mesmo quando a cepa selvagem não parece ser afetada pelo composto testado (WILLIAMS et al., 2011).

3.17.3. Análise dos mutantes preditos

3.17.3.1. Método 50:50

Para análise das vias afetadas a partir dos resultados obtidos pelos ensaios de interação genética, foi proposto a construção de um triplo mutante hipersensível, de

genótipo ΔPDR1ΔPDR3ΔSNQ2 através do novo método 50:50 (HORECKA; DAVIS, 2014). Um dos mecanismos de resistência a multidrogas em células eucariotas é o efluxo ativo de uma ampla variedade de xenobióticos através de transportadores ABC (*ATP-binding cassette*), entre eles a subfamília PDR, que está bem caracterizado como Pdr5p e Snq2p, localizados na membrana plasmática e realizam o efluxo de um amplo espectro de compostos. Ambas quando superexpressas conferem fenótipo de resistência e são controladas pelos fatores de transcrição Pdr1p e Pdr3p (PIECUCH; OBŁĄK, 2014). A obtenção do triplo mutante com fenótipo hipersensível possibilita um estudo mais específico dos genes pertencentes a via afetada pelo composto, já que o efluxo, que confere resistência à levedura, não funciona neste mutante, facilitando o entendimento da interação dos compostos com o genoma da levedura sem a interferência dos mecanismos naturais de defesa da levedura.

Inicialmente um *forward primer* foi construído com 50 pb acima do *start codon* e mais 50 pb abaixo *stop codon* do gene de interesse, com a parte homóloga U2 ao plasmídeo pJH140 (miniprep cepa JHB363) com a marca de seleção URA3. O *reverse primer* foi constituído por 50 pb abaixo *start codon* mais a região homóloga do plasmídeo (D2 e Sce)(figura 3). Para cada gene foram construídos *primers* específicos e assim foram obtidos três fragmentos diferentes, utilizados posteriormente nas transformações.

Para isso foi utilizado 1 µL de cada *primer* a 10 µM, 1 µL do plasmídeo pJH140, 1 µL de dNTP (Roche) a 10 mM, 5 µL de *Expand High Fidelity buffer* (10x com $MgCl_2$ - Roche) e 40,25 µL de água destilada estéril, acrescido de 0,75 µL da enzima *Expand HF* a 250 U (Roche). As condições da PCR foram: 2 minutos a 94 ^{o}C (inicial desnaturação), seguido de 30 ciclos de 30 segundo a 94 ^{o}C (desnaturação), 30 segundos a 52 ^{o}C (anelação), 2 minutos e 30 segundos a 72 ^{o}C (extensão). A extensão final foi realizada com 10 minutos a 72 ^{o}C.

Em 2 µL do produto de PCR foram adicionados 2 µL *6xOrange DNA loading Dye* (Thermo Scientific) e 8 µL de água destilada estéril, e submetidos a eletroforese em gel de agarose 1 % (*Ultra Pure* – Invitrogen) adicionado de *Syber safe DNA Gel Stain* (Invitrogen) por 30 minutos a 120 volts. O tamanho esperado do fragmento foi de 2.300 pb.

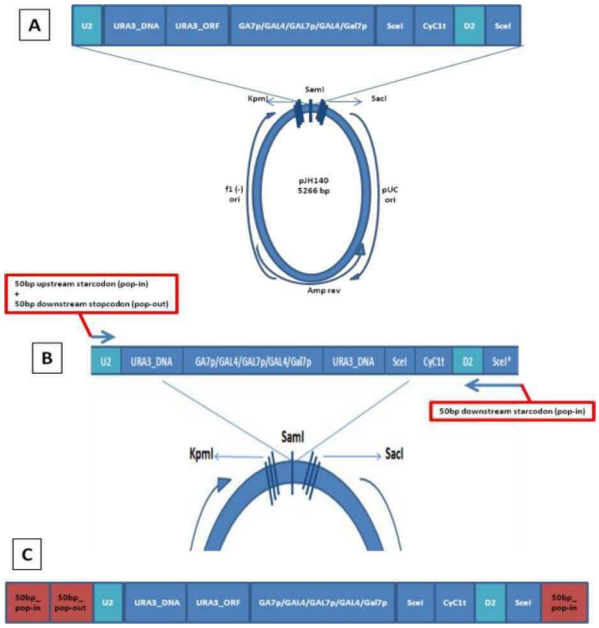


Figura 3 - A: Região do plasmídeo com a marca de seleção. B: Iniciadores na região homóloga a ser amplificada. C: Fragmento final.

Uma colônia isolada da levedura selvagem de *S. cereviseae* Rosetta BY4741 (mata; his3^1 leu2^0 ura3^0 met15^0) foi inoculada em 5 mL de YEPD e incubada a 30 ^{o}C por 16 horas a 150 RPM. A suspensão resultante foi diluída 1:10 em YEPD e a D.O. (densidade óptica) foi ajustada para 0,2 em 50 mL de YEPD em um comprimento de onda de 600nm. A suspensão foi incubada por 5 horas a 30 ^{o}C sob agitação até a D.O. de 1,0. Em seguida 50 mL foram centrifugados a 4000 RPM por 5 minutos em temperatura ambiente. O sobrenadante foi removido e o pellet foi lavado com 20 mL de água destila estéril. Uma nova centrifugação foi realizada e o pellet foi ressuspendido em 1 mL de água e transferido para um microtubo e submetido a uma nova centrifugação. O pellet foi ressuspendido em 1 mL da solução estéril TE/LiOAc e novamente centrifugado.

As células foram ressuspendidas em 0,250 mL da mesma solução, obtendo-se assim células competentes. Uma mistura de 10 µL de DNA (*salmon sperm* – Sigma), 20 µL do produto de PCR e 50 µL de células competentes foram adicionados a 1 mL de PLATE (800 µL 50 % PEG 4000, 100 µL 10X TE e 100 µL 10x LiOAc) e incubadas em banho seco por 1 hora a 30 ^{o}C. Em seguida foi realizado um choque térmico a 42 ^{o}C por 15 minutos. As células foram lavadas duas vezes com água destilada estéril (4000 RPM por 1 minuto) e gentilmente ressuspendidas em 1 mL de YEPD e incubadas por 1 hora e 50 minutos a 30 ^{o}C. As células foram lavadas duas vezes e ressuspendidas em 200 µL de 1x TE e plaqueadas em duas placas com meio SD-URA (*Yeast minimal media / Synthetic defined without uracil*) com pérolas de vidro. As placas foram incubadas a 30 ^{o}C por 48-96

horas. Neste passo (figura 4) a região homóloga do fragmento e do gene no genoma sofrem recombinação, levando a integração do fragmento com uma marca de seleção. De duas a três colônias foram obtidas e estriadas em meio SD-URA a 30 ^{o}C por 48 horas para a replicação de mais colônias transformadas.

Uma colônia isolada foi inoculada em 5 mL de YEPD a 30 ^{o}C por 16 horas com agitação. O DNA genômico foi purificado das células recuperadas da suspensão usando o kit *Master Pure Yeast DNA Purification* (Epicenter-MPY80200).

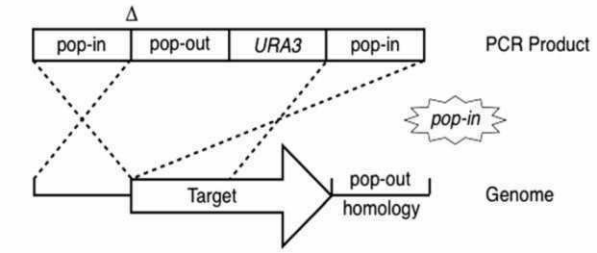


Figura 4 - Esquema da recombinação da região homóloga do fragmento e do gene no genoma, levando a integração do fragmento com uma marca de seleção (imagem: (HORECKA; DAVIS, 2014).

A placa com as colônias foi mantida a 4 ^{o}C até a confirmação por PCR. Para isso foi utilizado 1 µL de cada *primer* de confirmação a 10 µM, 1 µL do DNA genômico, 1 µL de dNTP (Roche) a 10 mM, 5 µL de *Expand High Fidelity* buffer (10x com $MgCl_2$ - Roche) e 40,25 µL de água destilada estéril, acrescido de 0,75 µL da enzima *Expand HF* a 250 u (Roche). As condições de PCR foram as mesmas da reação realizada anteriormente, assim como a visualização da banda em gel de agarose 1 %.

Os *primers* de confirmação foram construídos, sendo o *forward* com 80 pb acima do *start codon* e 20 pb do gene a ser deletado, e o *reverse* com uma região fixa codificante da sequência do gene URA3. A cepa selvagem foi utilizada como controle. O tamanho do produto de PCR esperado foi de 906 pb e a não visualização da banda para a cepa selvagem. Os *primers* utilizados para a próxima etapa de confirmação também foram utilizados como controle, com tamanho esperado de 552 pb. Com a confirmação da inserção do fragmento no DNA genômico, colônias foram estriadas separadamente em placas com YEPgal e incubadas a 30 ^{o}C por 48 horas (figura 5). Esta passagem aumenta a probabilidade de uma recombinação entre uma região do fragmento inserido e uma região homóloga no DNA genômico. Após 48 horas de incubação, colônias positivas foram estriadas por duas vezes em placas com SD+5-FOA (5-Fluoroorotic Acid) por 48-72 horas cada. As células que não realizaram a recombinação, e assim não excluíram o fragmento

com URA3, convertem o 5-FOA (presente no meio) em fluorodeoxiuridina, o qual é tóxico para as células, ou seja, o sucesso da recombinação é avaliado pela capacidade da levedura de crescer neste meio.

Uma colônia foi crescida em meio líquido YEPD e o mesmo protocolo de confirmação utilizado anteriormente foi seguido, porém foram utilizados *primers* que se alinham a região do gene que foi retirada após a recombinação. Neste sentido, foi esperado um tamanho de produto de 552 pb para a cepa selvagem e nenhuma banda para a cepa mutante.

A cada transformação, uma colônia foi incubada em 3 mL de YEPD a 30 ^{o}C por 16 horas a 150 RPM. Foram feitas alíquotas de 500 µL acrescidas de 500 µL de glicerol 40 % em microtubos e mantidas a – 80 ^{o}C.

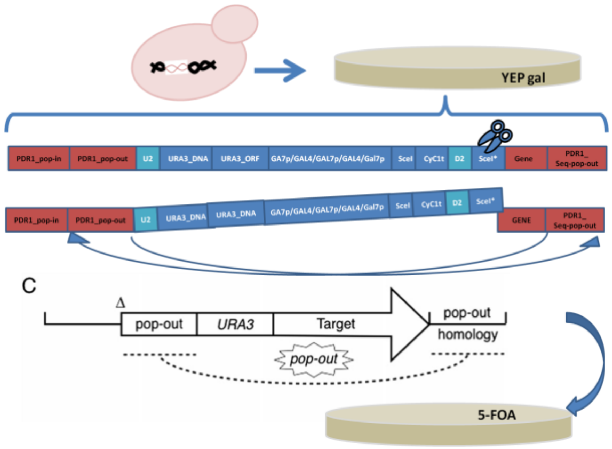


Figura 5 - Esquema recombinação entre uma região do fragmento inserido e uma região homóloga no DNA genômico.

Desse modo, o mutante PDR1 foi feito a partir da cepa selvagem. Com este mutante, conforme descrito anteriormente, foi feita a deleção do gene PDR3, gerando um duplo mutante, a partir do qual foi feito a deleção do gene SNQ2, obtendo-se assim o triplo mutante ΔPDR1ΔPDR3ΔSNQ2.

Com este mutante, foi proposto que uma sequencia GFP fosse inserida no genoma do triplo mutante junto aos genes de interesse, ou seja, aos genes pertencentes à via afetada pelo composto (galato de decila), possibilitando assim a análise da influência do composto na expressão deste gene pela intensidade de fluorescência emitida pela GFP.

Para isso, seriam construídos iniciadores referentes ao plasmídeo contendo a sequência GFP e uma marca de seleção HIS (plasmídeo pA/B GFP HIS *foward*: GGTGACGGTGCTGGTTTA, *reverse*: GATATCATCGATGAATTCG), além da região homóloga ao gene de interesse (45 pb *dow*/45 pb *up*). O protocolo de transformação seria

seguido como descrito anteriormente, porém as leveduras transformadas seriam plaqueadas em SD-HIS.

Para confirmação da inserção da sequencia GFP, as condições de PCR seriam: 5 minutos a 94 ^{o}C seguido de 30 ciclos de 30 segundo a 94 ^{o}C, 30 segundos a 52 ^{o}C, 4 minutos a 68 ^{o}C. A extensão final será de 5 minutos a 68 ^{o}C. O tamanho do fragmento esperado seria de 2590 pb, e para a confirmação seriam utilizados *primers* que anelassem a uma região interna do gene com um tamanho esperado de 250 a 300 pb.

Desta maneira cepas hipersensíveis (triplo mutante) marcadas com GFP para o gene de interesse seriam construídas para uma análise final dos dados obtidos neste projeto. Durante meu doutorado sanduiche no laboratório do Prof. Charles não foi possível realizar esta análise com os vários genes preditos. Porém, este mutante esta em nosso laboratório, e de acordo com os resultados obtidos a seguir, esta análise poderá ser realizada posteriormente.

3.17.3.2. Cepa GFP x galatos

O laboratório do Prof. Dr. Charles Boone tem uma coleção da levedura *S. cereviseae* com genes comumente estudados anexados a uma sequencia GFP, porém não possui um genótipo hipersensível (como o mutante construído anteriormente). Como visto em resultados, o composto influencia na via da n-glicosilação ou na resposta ao não enovelamento de proteínas. Neste sentido, foi analisada desta coleção a cepa chamada UPR (*unfold protein response*), na qual a sequência GFP está adjacente aos genes IRE1, ATF6 e PERK. Juntos estes sensores coordenam uma série de respostas conhecidas como resposta a proteínas não enoveladas, a qual é uma via de transdução de sinal que mantém a homeostase do retículo endoplasmático.

A cepa foi mantida em YEPD com glicerol 40 % em microtubos a – 80 ^{o}C e plaqueadas em YEPD, incubadas por 48 horas a 30 ^{o}C. Uma colônia foi cultivada em 2 mL de meio SD DO-Trp por 18 horas, 30 ^{o}C a 150 RPM. A suspensão foi diluída 1:10 em SD DO-Trp, com volume final de 12 mL e incubada por 3 horas (fase log), 30 ^{o}C a 150 RPM. A suspensão foi dividida em 10 tubos com 1 mL de SD DO-Trp para o tratamento, com 22,5 μM de cada galato (nonila, decila, undecila e dodecila), mais o controle com quantidade referente de DMSO. Os tubos foram incubados por 3 e 24 horas, 30 ^{o}C a 150 RPM. Para a análise, a cada tempo a suspensão foi centrifugada a 10000 RPM por 1 minuto. Foram recolhidos 0,50 μL da porção entre o *pellet* e o sobrenadante, e depositados cuidadosamente em uma lâmina de vidro contendo uma película de ágar a 2 %, e coberta

por uma lamínula de vidro retangular. Previamente a lâmina com ágar foi montada e dividida em secções para cada amostra (figura 6).

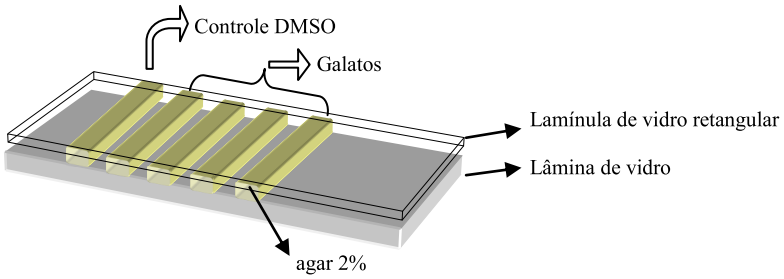


Figura 6 - Esquema para análise das cepas GFP.

3.17.4. Avaliação da n-glicosilação

Diferentes trabalhos na literatura apresentam a influencia da n-glicosilação na patogenicidade de fungos. A n-glicosilação é um processo fundamental para o correto funcionamento de importantes proteínas/enzimas, tais como relacionados à manutenção da parede celular. Se o processo de n-glicosilação é defeituoso, pode ocorrer um acúmulo de proteínas defeituosas, as quais em níveis elevados são tóxicas para o organismo, levando a ativação da via de resposta a proteínas não enoveladas para um possível reparo.

3.17.4.1. Ensaio enzimático

Este teste foi realizado em colaboração com o Dr. Fausto Almeida, do Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, da Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo.

3.17.4.1.1. Preparo da amostra

A massa fúngica do meio Fava-Netto dos fungos Pb18 e Pl01 foram transferidas para um erlenmeyer com meio BHI e incubado por 3 dias, a 37^{o}C, 150 RPM. Após o tempo de incubação 10^6 células/mL foram inoculadas em 200 mL de YEPD líquido. O tratamento foi realizado com a concentração final de 0,5µg/mL do composto galato de decila e o controle com DMSO. Ambos foram incubados por 3 dias, a 37^{o}C, 150 RPM. A suspensão fúngica foi centrifugada a 4000 RPM, por 10 minutos a 4 ^{o}C.

O pellet obtido foi lavado duas vezes com PBS contendo 1mM de PMSF (fenil metil sulfonil fluoreto). As células foram rompidas por sonicação (3 pulsos a cada 60 segundos) seguido por centrifugação a 10000 RPM, por 15 minutos a 4 ^{o}C, sendo que o extrato bruto foi obtido do sobrenadante. A concentração de proteínas foi quantificada pelo

Bicinchoninic Acid Assay (Pierce Chemical Co., Rockford, IL, USA), com soro albumina bovino como padrão (DOS REIS ALMEIDA et al., 2010).

3.17.4.1.2. Avaliação da atividade enzimática

A atividade da α-(1,4)-amilase foi analisada pelo monitoramento da hidrolise de amido de acordo com o método de Fuwa (1954). Uma unidade de atividade da α-(1,4)-amilase foi descrita como uma quantidade de enzima requerida para hidrolisar 0,1mg de amido por minuto. A atividade da α-(1,3)-glucanase foi determinada como descrito por Dos Reis Almeida et al (2014), usando α-glucano de *Aspergillus niger* como substrato. Uma unidade de atividade da α-(1,3)-glucanase foi definida como a quantidade de enzima requerida para levar a 1 μM de redução de açúcares por minuto. A atividade da N-Acetyl-β-D-glucosaminidase foi verificada pelo monitoramento de taxa de formação de *p*-nitrofenol de *p*-NPGlcNAc (Sigma) (DOS REIS ALMEIDA et al., 2011; ALMEIDA et al., 2015). Uma unidade da atividade de N-Acetyl-β-D-glucosaminidase foi definida como a quantidade de proteínas requeridas para produzir 1 μM de *p*-nitrophenol em 1 minuto a 37^{o}C. Os valores de atividade enzimática correspondem as medias dos valores de ao menos três replicas. A concentração de proteínas foi analisada usando *Bicinchoninic Acid Assay* (Pierce Chemical Co., Rockford, IL, USA), com soro albumina bovino como padrão.

3.17.5. Análise da expressão diferencial de genes

3.17.5.1. Extração de RNA total

A massa fúngica do meio Fava-netto dos fungos Pb18 e Pl01 foram transferidas para um erlenmeyer com meio BHI e incubado por 3 dias, 37^{o}C a 150 RPM. Após o tempo de incubação 10^6 células/mL foram inoculadas em 200 mL de YEPD líquido. O tratamento foi realizado com a concentração final de 0,5μg/mL do composto galato de decila e o controle com DMSO. Ambos foram incubados por 3 dias, 37^{o}C a 150 RPM. Após este período a suspensão fúngica foi centrifugada a 5000 RPM, por 10 minutos a 4 ^{o}C.

O pellet foi ressuspendido em PBS e 3 alíquotas de 600 μL em microtubos foram reservadas. As células foram centrifugadas a 5000 RPM por 1 minuto, a 4^{o}C e o sobrenadante foi descartado. Para todas as amostras, foi obtido um pellet de 100 mg de massa. Para a lise celular e extração do RNA total, o pellet foi ressuspendido em 600 μL Trizol (Ambion/RNA-LifeTechnologies) em temperatura ambiente (responsável por conferir a fase aquosa), homogeneizado e submetido ao vortex rigorosamente por 1

minuto. Os microtubos foram submetidos a banho-seco a 65 ^{o}C por 20 minutos, e logo em seguida incubados por 1 hora a -80^{o}C.

Para a purificação da amostra um volume de 200 µL de clorofórmio (Synth) (confere a fase orgânica) por microtubo foram adicionado. Os microtubos foram homogeneizados e centrifugados a 13000 RPM por 10 minutos, a 4^{o}C. A fase aquosa foi transferida a um novo microtubo, e 100 µL de trizol e mais 50 µL de clorofórmio foram adicionados por microtubo. Estes foram homogeneizados e centrifugados a 13000 RPM por 10 minutos, a 4^{o}C. Este último passo foi realizado 2 vezes ou mais. O RNA tem maior propensão para a fase aquosa, enquanto que os restos celulares ficam na fase orgânica. Para uma última etapa de purificação, 200 µL de clorofórmio foram adicionados e submetidos a uma centrifugação a 13000 RPM por 10 minutos, a 4^{o}C. O sobrenadante foi transferido a um novo microtubo, no qual RNA ficará reservado.

A cada microtubo foram adicionados 700 µL de isoproponol (Fmaia) e 11,2 µL de glicogênio 20mg/mL (2µL a cada 125 µL de isopropanol) e acondicionados a -80 ^{o}C por 20 minutos ou *overnight*. O pellet foi obtido e reservado após uma centrifugação a 13000 RPM por 20 minutos, a 4^{o}C. Duas lavagens foram realizadas com 1000 µL de etanol a 70%, e centrifugações a 13000 RPM, por 5 minutos, a 4^{o}C. O resíduo de etanol foi eliminado submetendo os microtubos ao aparelho *Concentrator plus* (eppendorf) 30^{o}C, 2-3 minutos, modo vac-alc.

3.17.5.2. Síntese de cDNA

A reação de primeira fita de cDNA foi através do kit *Smart* (Clonetech Laboratories). Neste processo foram utilizados 1 µg de RNA total, e a essa quantidade foram adicionados 2 µL do *primer* dT12-18 (10 pmol/µL) (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3') e essa mistura foi incubada por 2 minutos a 72 ^{o}C. Em seguida foram acrescentados 2 µL do *primer* CDS (10 pmol/µL) (5'-AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$$N_1N$, onde N=A, C, G ou T; N_1=A, G ou C), 4 uL tampão da transcriptase reversa 5X; 2 µL DTT 100mM; 2 µL dNTPs 10mM cada; 2 µL de RNA-out; 1,2 µL de MgCl2; 200U da enzima transcriptase reversa (SuperScript II, Invitrogen). Esta reação foi incubada a 42 ^{o}C durante 1 hora e trinta minutos. Após este período foi adicionado 80 µL de TE (10 mM Tris-HCl; 1 mM EDTA pH 8,0) e aquecida por mais 7 minutos a 72^{o}C.

3.17.5.3. PCR em tempo real

PCR em Tempo Real foi utilizado para avaliar a expressão relativa dos genes que codificam α-(1,3)-glucanase (AGN), α-(1,3)-glucano sintase (AGS), N-Acetyl-β-D-glucosaminidase (NAG), α-amilase (AMY), e os genes sensores de estresse ao retículo endoplasmático IRE1 e HAC. O gene endógeno utilizado como controle foi a α-tubulina, conforme tabela abaixo (tabela 1). As reações foram realizadas utilizando-se 1 µL de cDNA, 10 µL de Máxima SYBR Green/ROX qPCR Master Mix (2 x) (Qiagen), e 0,5 µM de cada primer, sendo adicionado a reação água DEPC até atingir o volume de 20 µL. As reações foram realizadas com temperatura inicial de 95 ºC por 1 minuto, seguido por 1 minuto a 95 ºC. Depois, 40 ciclos de 5 segundos a 95 ºC, seguidos de anelamento e síntese a 60 °C por 32 segundos. Em seguida a PCR, a análise da curva de *melting* foi realizada, na qual foi confirmada a emissão de sinal correspondente a um único produto. As reações foram realizadas em triplicata com o aparelho *Applied Biosystems* 7500 *cycler*. Os cálculos foram validados pela análise da eficiência da amplificação do gene de interesse e de controle em diferentes concentrações da amostra. Os dados foram analisados usando o método $2^{-\Delta\Delta CT}$ (LIVAK; SCHMITTGEN, 2001). Como controle negativo foi utilizado todos os reagentes exceto a amostra sendo substituída por água DEPC, para o qual depois de 40 ciclos de amplificação, nenhum produto de amplificação foi detectado.

Tabela 1 - *Primers* específicos para genes codificantes (F: *foward,* R: *reverse*).

ID genes Pb18	*Primers*		ID genes Pl01	*Primers*
Pb18NAG1_F	TCATGCAGAGCTGGAACAAC		Pl01NAG1_F	TCATGCAGAGCTGGAACAAC
Pb18NAG1_R	CATTGTAGCGGTGGTCATTG		Pl01NAG1_R	CATTGTAGCGGTGGTCATTG
Pb18AGN_F	CAACCATGGTCAGCAAACAC		Pl01AGN_F	CAACCATGGTCAGCAAACAC
Pb18AGN_R	CTGTAGCCCTGAACCCACAT		Pl01AGN_R	CTGTAGCCCTGAACCCACAT
Pb18AMY_F	CGGTGACTTGTATGGCCTTT		Pl01AMY_F	CGGTGACTTGTATGGCCTTT
Pb18AMY_R	CAGTTGGCCATTACATGACG		Pl01AMY_R	CAGTTGGCCATTACATGACG
Pb18IRE1_F	GGAGCTTGCGAAACAGAAAC		Pl01IRE1_F	CCCCCAGAGATATCGTGAGA
Pb18IRE1_R	TTGGATGGAACCCTTGTCTC		Pl01IRE1_R	TTAGAACGTGGGGCCTAATG
Pb18HAC1_F	CTTGTCCCCATCCTCTTCAA		Pl01HAC1_F	CTTGTCCCCATCCTCTTCAA
Pb18HAC1_R	TTCAAGGATCCCTCCAACTG		Pl01HAC1_R	TTCAAGGATCCCTCCAACTG
Pb18AGS1_F	CTACAACCACCAGCCACCTT		Pl01AGS1_F	CTACAACCACCAGCCACCTT
Pb18AGS1_R	TTCGAGTTCAACGCACTGTC		Pl01AGS1_R	TTCGAGTTCAACGCACTGTC
Pb18tub_F	GGGAGTTGTATTGCCTGGAA		Pl01tub_F	CTGTTACCAGCCTCCCCATA
Pb18tub_R	AGCTTTCCGTTCCTCAGTCA		Pl01tub_R	TGCTTCAGAAATGGCAGTTG

4. RESULTADOS

4.1. Quantificação da concentração de nitritos no sobrenadante da cultura de macrófago RAW após contato com o composto G14.

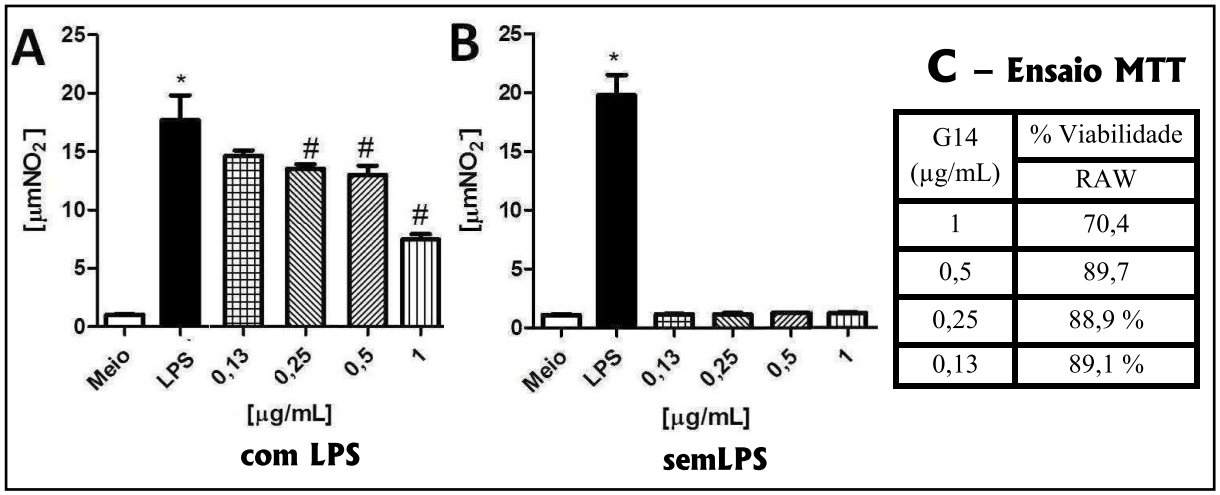
O óxido nítrico (NO) e espécies reativas de oxigênio afetam virtualmente cada etapa do desenvolvimento da inflamação. A atividade antioxidante do galato de decila (G14) sugere uma um potencial anti-inflamatório, e para esta confirmação foi utilizado como modelo experimental de inflamação o macrófago (RAW), cuja estimulação com LPS desencadeia a produção de óxido nítrico. Para tanto, foram usadas as concentrações de 0,13 a 1 μg/mL, com ou sem estímulo por LPS.

Na figura 7A estão representados os resultados dos macrófagos estimulados com LPS, e na figura 7B, sem estímulo, além dos controles somente meio (meio de cultura e monocamada celular – barra branca) e LPS (meio de cultura acrescido de 20 μL de LPS a 1 mg/mL sob a monocamada celular, sem tratamento com G14 – barra preta).

Na figura 7 A, as concentrações de 0,25 a 1 μg/mL, apresentaram diferença estatística em relação ao controle LPS na quantificação de nitritos no sobrenadante. Na concentração de 1 μg/mL houve uma redução de quase 50 % da concentração de nitrito em relação ao controle, enquanto que nas concentrações de 0,25 e 0,5 μg/mL houve redução de cerca de 20 % em relação ao controle. Estes dados sugerem que o G14 é capaz de diminuir a concentração final de nitritos, derivados estes do óxido nítrico produzido pela linhagem de macrófagos RAW, quando estes são estimulados por LPS. A concentração de 0,13 μg/mL, estatisticamente, não apresentou diferença em relação ao controle LPS.

Na figura 7B nenhuma concentração do G14 testada apresentou diferença estatística em relação ao controle do meio (barra branca). Este dado sugere que o G14 não tem capacidade de estimular a produção de óxido nítrico em macrófagos RAW quando estes não são estimulados por LPS.

A citotoxicidade do G14 nas concentrações entre 0,13 e 1 μg/mL para as células RAW foi avaliado para monitorar a influência da viabilidade da célula na produção de óxido nítrico. Na concentração de 1 μg/mL foi observado 70,4 % de viabilidade celular, e para as outras concentrações uma média de 89 % de viabilidade (figura 7C). Estes dados inferem que a perda da viabilidade celular na concentração de 1 μg/mL seja responsável pelo baixo valor da quantificação de óxido nítrico nesta concentração.



C – Ensaio MTT

G14 (µg/mL)	% Viabilidade RAW
1	70,4
0,5	89,7
0,25	88,9 %
0,13	89,1 %

Figura 7 - Avaliação do potencial antioxidante do G14: (A/B) monocamada de macrófago peritoneal murino RAW desafiada com G14 na faixa de concentração de 1 a 0,13 µg/mL. (A) com estimulação de LPS (1 mg/ml), (B) sem estimulação por LPS. Barra branca: macrófagos não tratados com G14 e não estimulados com LPS. Barra preta: macrófagos não tratados com G14 e estimulados com LPS (1 mg/ml). (C) Taxa de viabilidade da linhagem de macrófago peritoneal murino RAW após 24 horas de contato de G14 na faixa de concentração de 1 a 0,13 µg/mL . *em relação ao controle Meio ($p<0,05$); # em relação ao controle de LPS ($p<0,05$). (ANOVA/Tukey's post-test).

4.2. Análise do composto G14 na atividade mitocondrial e do desenvolvimento das células fúngicas de *P. lutzii*.

Para determinar a concentração que inibe o desenvolvimento de 10^5 células fúngicas de *P. lutzii* por poço, foi aplicado o ensaio de MTT (figura 8A). Todas as concentrações depois de 24 e 48 horas de contato com as células fúngicas apresentaram diferença estatística ($p<0,05$) em relação ao controle positivo (sem o composto G14). Não houve, para os dois tempos testados, diferença estatística entre as concentrações. Em média, houve uma redução de 42 % da atividade metabólica entre o controle e as concentrações depois de 24 horas de contato, e uma redução de 65 % depois de 48 horas.

A influência do G14 foi, também, avaliada quanto a formação de novos brotamentos para a análise do desenvolvimento das células fúngicas (figura 8B). No tempo T0, foi observado no controle positivo que em torno de 50 % das células fúngicas possuíam brotamento. Nos tempos T24 e T48 de incubação foi observado no controle positivo cerca de 90 % de células fúngicas com brotamentos.

Ao analisarmos as células fúngicas que estavam em contato com as diferentes concentrações de G14, foi observado que em torno de 51 % das células fúngicas possuíam brotamentos nas concentrações de 0,25 e 0,5 µg/mL em T24, indicando que cerca de 38,5 % da células fúngicas tiveram a formação de novos brotamentos inibidos, enquanto que a

concentração de 0,13 µg/mL com 63,65 % das células fúngicas com brotamento, apresentou uma inibição em torno de 26 %.

Em T48 a concentração 0,5 µg/mL não apresentou diferença estatística em relação a T24, apresentando ainda cerca de 40 % de inibição para a formação de novos brotamentos. As concentrações 0,25 e 0,13 µg/mL apresentaram um aumento no número de brotamentos com 61 % e 77 %, respectivamente, das células fúngicas com brotamentos, apresentando, em relação ao controle positivo, inibição para a formação de novos brotamentos de 29 e 13 %, respectivamente.

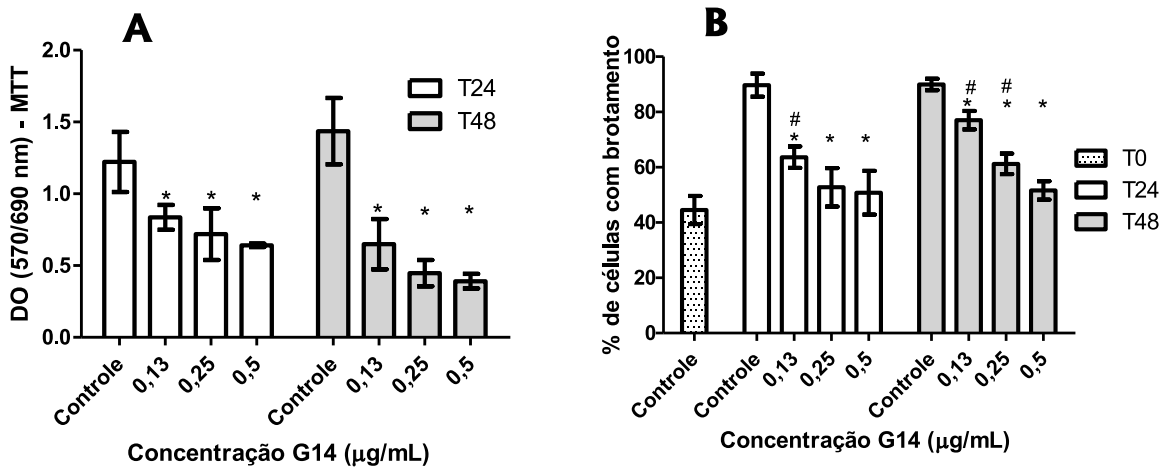


Figura 8 - Análise da atividade mitocondrial e do desenvolvimento das células fúngicas de *P. lutzii* após 24 e 48 horas de contato com as concentrações de 0,5 a 0,13 µg/mL do composto galato de decila. A: ensaio de MTT para análise da atividade mitocondrial; B: porcentagem de células com brotamentos para analise do desenvolvimento. Barra controle: células fúngicas avaliadas sem contato com G14 em cada tempo. *p<0,05 em relação ao controle do tempo. # p<0,05 em relação à condição tratado 0,5 µg/mL. (ANOVA/Tukey's post-test).

4.3. Perfil de interação e influência do composto G14

O perfil de interação do fungo *P. lutzii* e as linhagens A549 e MRC-5, os macrófagos AMJ2-C11 e os hemócitos de *G. mellonella* foram analisados por citometria de imagem e citometria de fluxo. Desse modo foi possível observar uma correlação de perfil de interação entre as linhagens celulares de pulmão e a espécie *P. lutzii*. Além disso, foi possível observar uma correlação de análise entre os dois métodos.

Na figura 9 (AB) foi observado um progressivo aumento na taxa de interação do fungo com as células A549 de até 6 horas (p<0,05) e após esse período verificou-se diminuição na interação (p<0,05). Para a linhagem MRC-5, a interação foi semelhante ao

observado para a linhagem A549. Um progressivo aumento na taxa de interação do tempo de 1 hora até 6 horas ($p<0,05$), e entre os tempos de 06 e 24 horas, queda na taxa foi observada ($p<0,05$) (figura 9D).

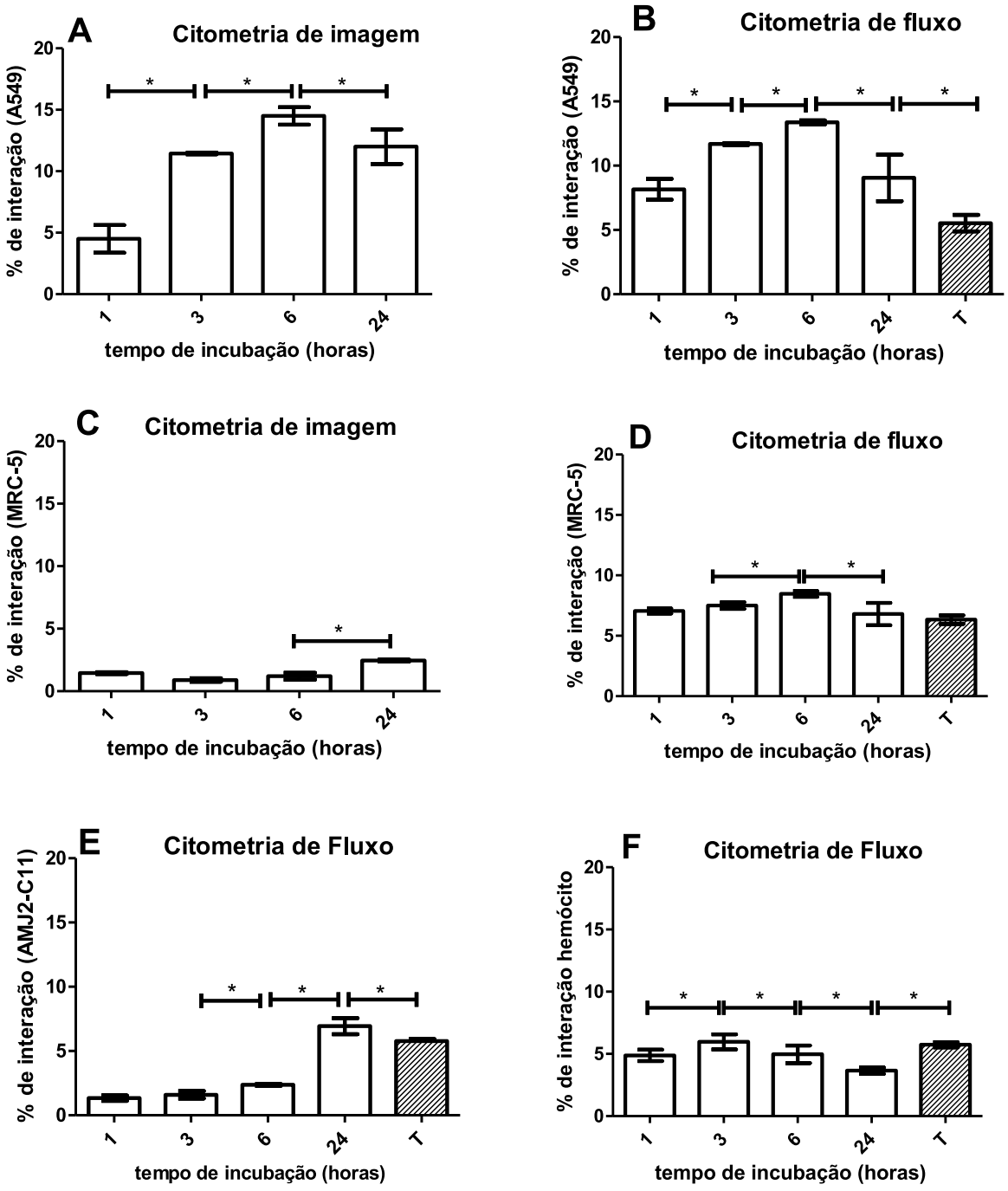


Figura 9 - Perfil de interação das células fúngicas de *P.lutzii* com as linhagens pulmonares humanas A549 (A, B) e MRC5 (C, D), a linhagem de macrófago alveolar murino AMJ2-C11 (E) e hemócitos da larva de *G. mellonella* (F) nos tempos de 1, 3, 6 e 24 horas por citometria de imagem (A,C) e de fluxo (B,D,E,F). Barra T: Taxa de interação após 24 horas de tratamento com G14 na concentração de 0,5 μg/mL por citometria de fluxo em comparação com a taxa de interação do tempo de 24 horas (B,D,E,F). *$p<0,05$ entre os tempos.

O perfil de interação para o macrófago AMJ2-C11 (figura 9E) foi analisado somente por citometria de fluxo e não foi observada diferença estatística entre o tempo de 01 e 3 horas. Porém, ao contrário do padrão antes observado para as duas linhagens, houve um aumento significativo entre os tempos de 06 e 24 horas ($p<0,05$).

Por citometria de fluxo foi observado o perfil de interação do fungo com os hemócitos presentes na hemolinfa da larva *G. mellonella* (figura 9F). O perfil foi semelhante ao das células com aumento de interação entre os tempos de 01 e 3 horas, e queda entre os tempos de 6 e 24 horas ($p<0,05$).

O efeito do tratamento do G14 na concentração de 0,5 μg/mL por 24 horas na taxa de interação foi realizado por citometria de fluxo. Para a linhagem A549 o tratamento apresentou uma redução de 1,6 vezes na taxa de interação, em relação ao tempo de 24 horas ($p<0,05$). Para a linhagem MRC-5, não foi observada redução na taxa de interação em relação ao tempo de 24 horas. Para o macrófago AMJ2-C11, após o tratamento houve diferença estatística na taxa de interação entre o tempo de 24 horas e o tratamento ($p<0,05$), aproximadamente redução de 1,2 vezes (figura 9E). Para o hemócito, foi observada a diferença estatística na taxa de interação em relação ao tempo de 24 horas, porém um aumento na taxa após o tratamento (figura 9F).

Concomitante ao teste de interação foi seguido o ensaio de MTT para o monitoramento da viabilidade das células em relação ao composto na concentração de 0,5μg/mL do composto G14 com 24 horas de incubação. A linhagem MRC-5 apresentou uma média de 80,4 % de viabilidade, enquanto que a linhagem A549 apresentou 73,1% e o macrófago AMJ2-C11 apresentou uma média de 88,1 %.

4.4. Aquisição de imagens *In Cell Analyzer*

As imagens da figura 10 foram obtidas no *In Cell Analyzer* 2000 (GE). As imagens indicam a interação das células fúngicas de Pl01 entre as linhagens pulmonares humana A549 e MRC-5, e entre os hemócito da larva *G. mellonella* com 6 horas de interação.

As populações foram distinguidas pela marcação fluorescente. As células A549, MRC-5 e hemócitos foram marcadas com faloidina-Alexa648 e representadas na figura pela coloração verde. As células fúngicas foram marcadas com CFDA-SE e representadas pela coloração vermelha. O núcleo das células A549 e MRC-5 foram marcados com DAPI e representados pela coloração azul.

As setas brancas foram posicionadas próximas as células fúngicas que aparentemente estão internalizadas nas células ou hemócitos. As setas vermelhas estão posicionadas próximas às células fúngicas que estão aparentemente aderidas as células ou hemócitos.

Com análise destas imagens foi possível observar e comprovar a capacidade do fungo *P. lutzii* em aderir ou internalizar nos tipos celulares estudados com 6 horas de interação, e assim sugerir este tempo de interação como ideal para testes com compostos com potencial antifúngico, como foi utilizado neste trabalho, o galato de decila (G14).

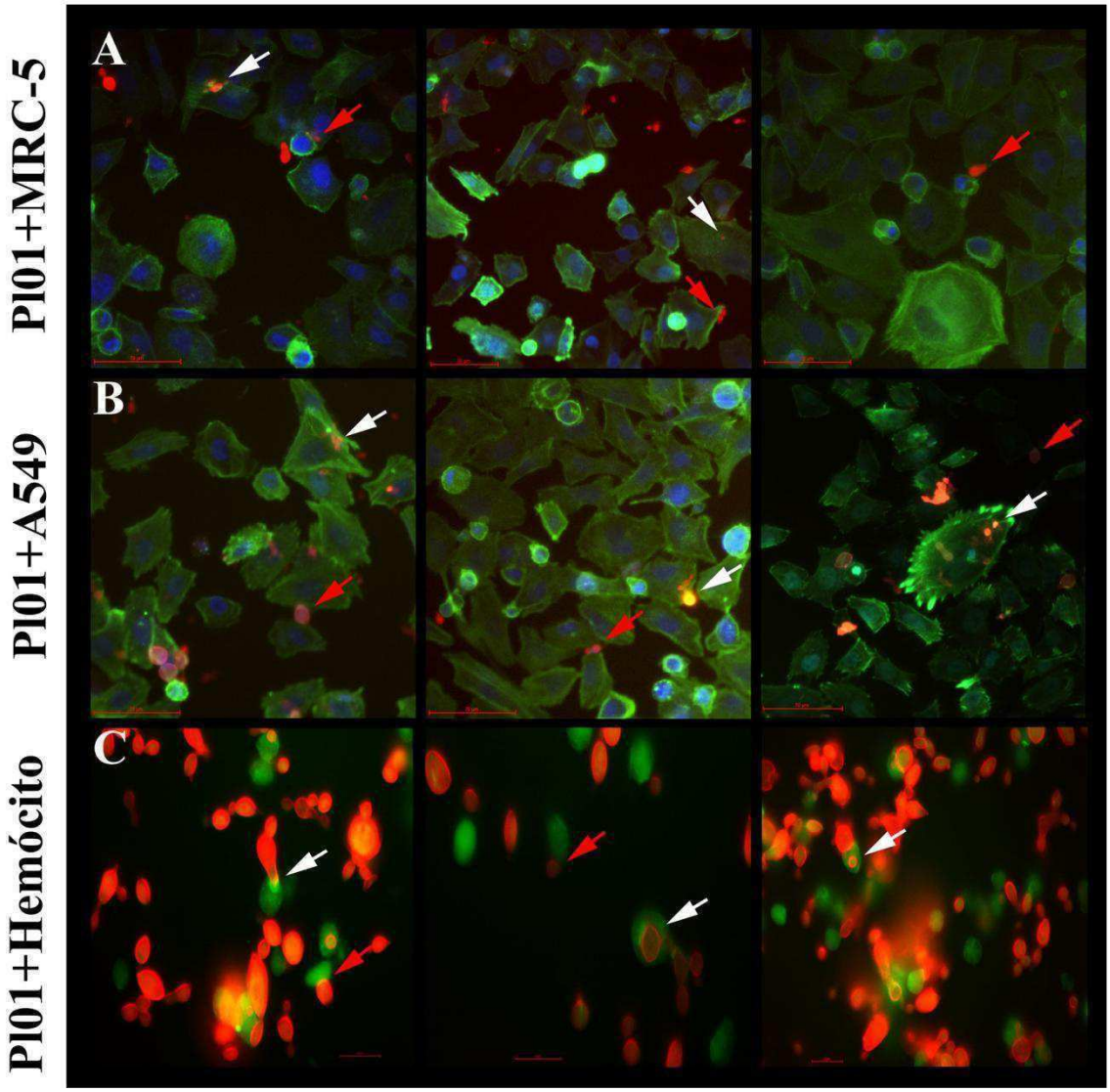


Figura 10 - Análise das imagens adquiridas no equipamento *In Cell Analyzer* 2000 da interação das células fúngicas de *P. lutzii* (Pl01) as linhagens de pulmonares humana A549 e MRC-5; e aos hemócitos da larva de *G. mellonella* após 6 horas de interação. (A) interação Pl01 e MRC-5; (B) interação Pl01 e A549; (C) interação Pl01 e hemócito. Seta branca: internalização do fungo ao tipo celular estudado; seta vermelha: fungo aderido ao tipo celular estudado. Células fúngicas marcada com CFDA-SE 15 µM (vermelho) e linhagem pulmonar e hemócitos marcados com Alexa Fluor® 647 faloidina 0,165 µM (verde). (A e B) núcleo das linhagens pulmonares marcado com DAPI.

4.5. Curva de sobrevivência

As larvas foram infectadas com *P. lutzii* na concentração de 10^6 fungos/larva. De acordo com a figura 11, a taxa de sobrevivência das larvas infectadas com Pl01 até o décimo dia foi de 50%.

Para o grupo controle PBS e grupo infectado/tratado observaram-se 75 % de sobrevivência das larvas após 10 dias de experimento. Este dois grupos apresentaram diferença estatística ($p<0,05$) em relação ao grupo infectado Pl01.

O grupo de larvas que foram inoculadas apenas com o composto apresentou queda na sobrevivência apenas no décimo dia, finalizando com cerca de 80 % de sobrevivência. Este grupo apresentou diferença estatística em relação ao grupo infectado Pl01 e não apresentou diferença em relação aos outros dois grupos.

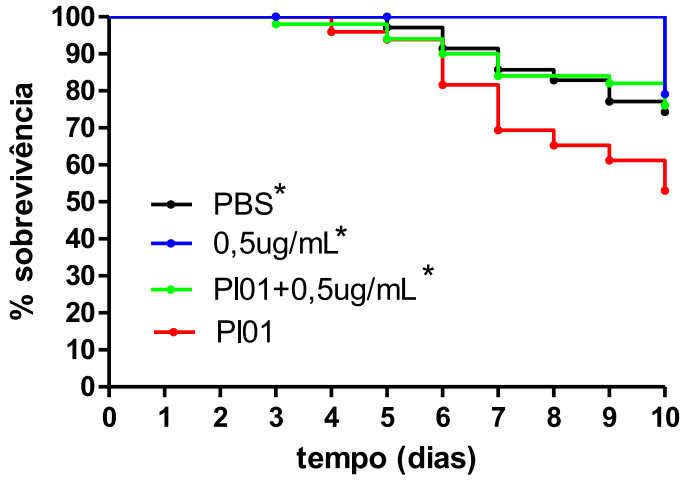


Figura 11 - Estudo da sobrevivência de *Galleria mellonella* após infecção com Pl01 e tratamento com G14 durante 10 dias de análise. Grupo controle PBS (linha preta), grupo infectado Pl01 (linha vermelha), grupo infectado Pl01 por seis horas e posteriormente tratado com dose equivalente a 0,5 µg/mL de G14 (linha verde), grupo de larvas inoculado com dose equivalente a 0,5 µg/mL de G14. * $p<0,05$ em relação ao grupo Pl01 (infectado).

4.6. Avaliação do mecanismo de ação

4.6.1. Ensaio químico-genético

A figura 12 apresenta as porcentagens de crescimento da coleção *Mini-pool* em relação ao controle DMSO, após 24 horas de contato com cada composto (ácido gálico e galatos) em diferentes concentrações. Foi aplicada uma escala de cores para diferenciar as porcentagens menores (vermelho) das maiores (verde).

Na primeira etapa da avaliação os compostos foram testados na faixa de concentração de 0,75 a 100 µM (figura 12A), e dentre eles foram selecionados quatro galatos (nonila, decila, undecila e dodecila), pois apresentaram taxa de crescimento de 50 a 80 % entre as concentrações de 25 e 12,5 µM. O ácido gálico e os outros galatos foram descartados por não apresentaram influencia no crescimento da coleção.

Na segunda etapa (figura 12B), os galatos selecionados foram testados na faixa de concentração de 12,5 a 50 µM, e a concentração de 22,5 µM foi escolhida para estes galatos, pois apresentou taxa de crescimento de 50 a 80 %. Todos os controles apresentaram perfil esperado nas duas etapas.

A

(µM)	AG	HEX	HEP	NON	DEC	UND	DODEC	TETRA		Control
100	104	52	6	7	6	6	7	14	102	DMSO
50	101	89	36	6	6	6	7	41	101	DMSO
25	104	95	80	21	14	37	38	83	101	DMSO
12.5	110	97	91	85	80	93	88	90	5	TUN(0.1mg/mL)
6.2	101	101	96	94	94	95	103	100	66	MMS(4%)
3.1	101	101	97	92	94	98	98	100	11	MICA(4.7µM)
1.5	102	111	102	95	111	102	100	102	55	BOR(1301µM)
0.75	103	107	100	102	99	99	105	101	85	BEN(1mg / mL)

B

(µM)		NON	NON	DEC	DEC	UND	UND	DODEC	DODEC
50	30	7	23	7	21	9	46	14	46
47.5	27.5	8	27	8	24	9	54	14	55
45	25	9	51	11	35	13	54	18	61
42.5	22.5	9	56	10	55	13	72	20	62
40	20	9	71	8	65	17	79	22	76
37.5	17.5	12	75	12	69	17	84	27	76
35	15	20	83	22	76	27	87	42	83
32.5	12.5	23	86	13	83	36	90	41	91

Figura 12 - Figura representativa da hipersensibilidade da coleção aos galatos em diferentes concentrações. A escala de cores indica a variedade da porcentagem de crescimento. VERDE: porcentagem de crescimento semelhante ao controle DMSO; VERMELHO: porcentagem de crescimento baixa em relação ao controle DMSO. (A): ácido gálico e os derivados hexila, heptila, nonila, decila, undecila, dodecila e tetradecila testados na faixa de concentração de 100 a 0,75 µM. (B): galatos selecionados nonila, decila, undecila e dodecila testados na faixa de concentração de 50 a 12,5 µM. Em destaque concentração selecionada: 22,5µM.

4.6.2. Sequenciamento plataforma *Illumina*

Através de PCR um marcador foi adicionado ao DNA genômico total de cada situação, conferindo uma identificação individual. Após eletroforese em gel de agarose 2% de cada produto, pode-se confirmar a integração do marcador ao fragmento a ser analisado, através da visualização de produto de 267 pb (Figura 13A). Em seguida, um volume de 10 µL do produto de PCR de cada condição foram reunidos e submetidos à nova eletroforese

em gel de agarose 2 % para posterior extração da banda com 267 pb contendo a biblioteca de DNA (Figura 13C).

A quantificação das cópias de DNA de cada mutante presente nas bibliotecas foi realizada de acordo com uma curva padrão presente no kit *Library Quantification* (*Illumina Sequencing Plataforms* – KAPA) com faixa de 20 a 0,0002 pM.

A quantificação de todas as replicas foram consideradas satisfatórias, uma vez que apresentaram entre 2 e 0,2 pM. De acordo com o kit, a concentração da amostra foi ajustada de acordo com o tamanho do fragmento, neste caso de 267 pb e com as diluições realizadas para a quantificação.

Em seguida a amostra foi diluída para 2000 pM de acordo com o kit *MiniSeq Reagent* v3 (*Illumina*) e enviada para sequenciamento.

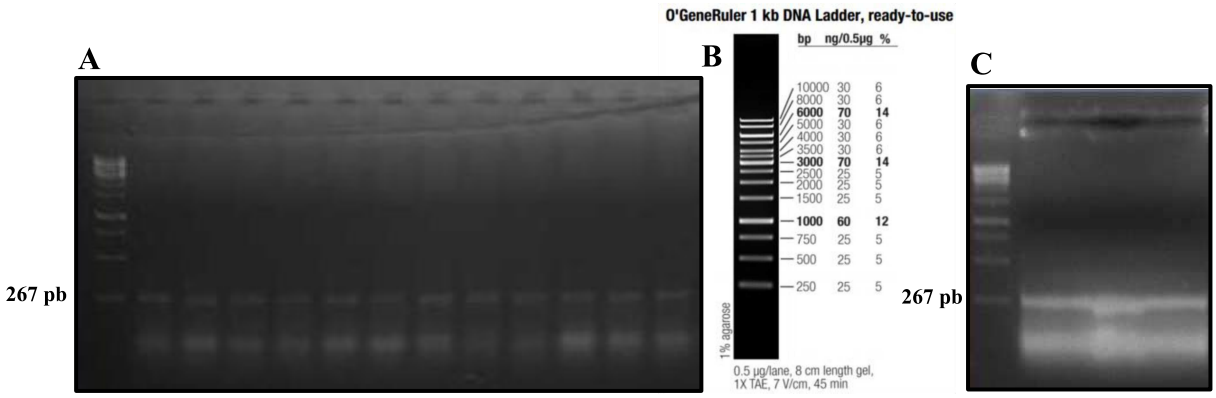


Figura 13 - A: Gel 2 % agarose para a confirmação do produto de PCR de cada condição contendo *barcode* do mutante mais a integração do marcador individual 10 pb. Tamanho produto final 267 pb. B: Padrão de 250 a 10000 pb (ThermoFicherScientific-SM1163). C: Exemplo gel 2 % agarose para extração da banda com 267 pb contendo a biblioteca de DNA.

Após o sequenciamento, os dados foram tratados pelo programa *Java Treeview heatmap*, o qual realiza a disposição dos dados por similaridade, tanto para o composto quanto para os mutantes. Tais dados, referentes ao número de cópias genômicas de cada mutante em relação aos compostos, quando comparados com o controle DMSO, são apresentados em uma escala de cores do vermelho, que indica um baixo número de cópias, um fenótipo de hipersensibilidade; ao verde, que indica um alto número de cópias, um fenótipo de hiper-resistência.

Dessa forma, mutantes que apresentaram um baixo número de cópias indicam que foram afetados pelo composto, indicando que a mutação presente, quando na presença do composto, confere sensibilidade ao mutante afetando assim seu crescimento. Já os mutantes

que apresentaram alto número de cópias indicam que podem ter resistência ao composto, indicando que aquela mutação não afeta o crescimento na presença do composto. Por esta análise, foi possível observar que um conjunto de 50 mutantes da coleção *Mini-pool* apresentaram perfil de hipersensibilidade aos compostos testados (galatos de nonila, decila, undecila e dodecila), revelando um perfil de interação Químico-genético. Foi possível observar também que dentro destes grupos os agentes bortezomib, benomyl e tunicamicina apresentaram grupos de mutantes hipersensíveis compatíveis aos dos compostos testados. Na figura 14 a região em destaque prioriza os mutantes que apresentaram hipersensibilidade para os galatos e para o agente tunicamicina (vermelho).

4.6.3. Interação genética

Os dados obtidos no sequenciamento foram comparados com um banco de dados de duplo mutantes. Os perfis dos mutantes que apresentaram hipersensibilidade aos galatos foram equiparados com os perfis dos duplos mutantes. Quando os perfis são semelhantes, o duplo mutante traz como informação o possível gene influenciado pelo tratamento, levando a dedução da provável via que os compostos podem afetar diretamente. As tabelas 2 e 3 apresentam os processos envolvidos com os duplos mutantes que apresentaram perfis semelhantes aos mutantes tratados com os galatos (tabela 2) ou com os agentes controles (tabela 3). Dessa forma os mutantes afetados pelos galatos estão envolvidos com a via de glicosilação e/ou a resposta a proteínas não enoveladas (tabela 2). O perfil de influência dos compostos galatos foi muito semelhante ao perfil apresentado pela tunicamicina (tabela 3).

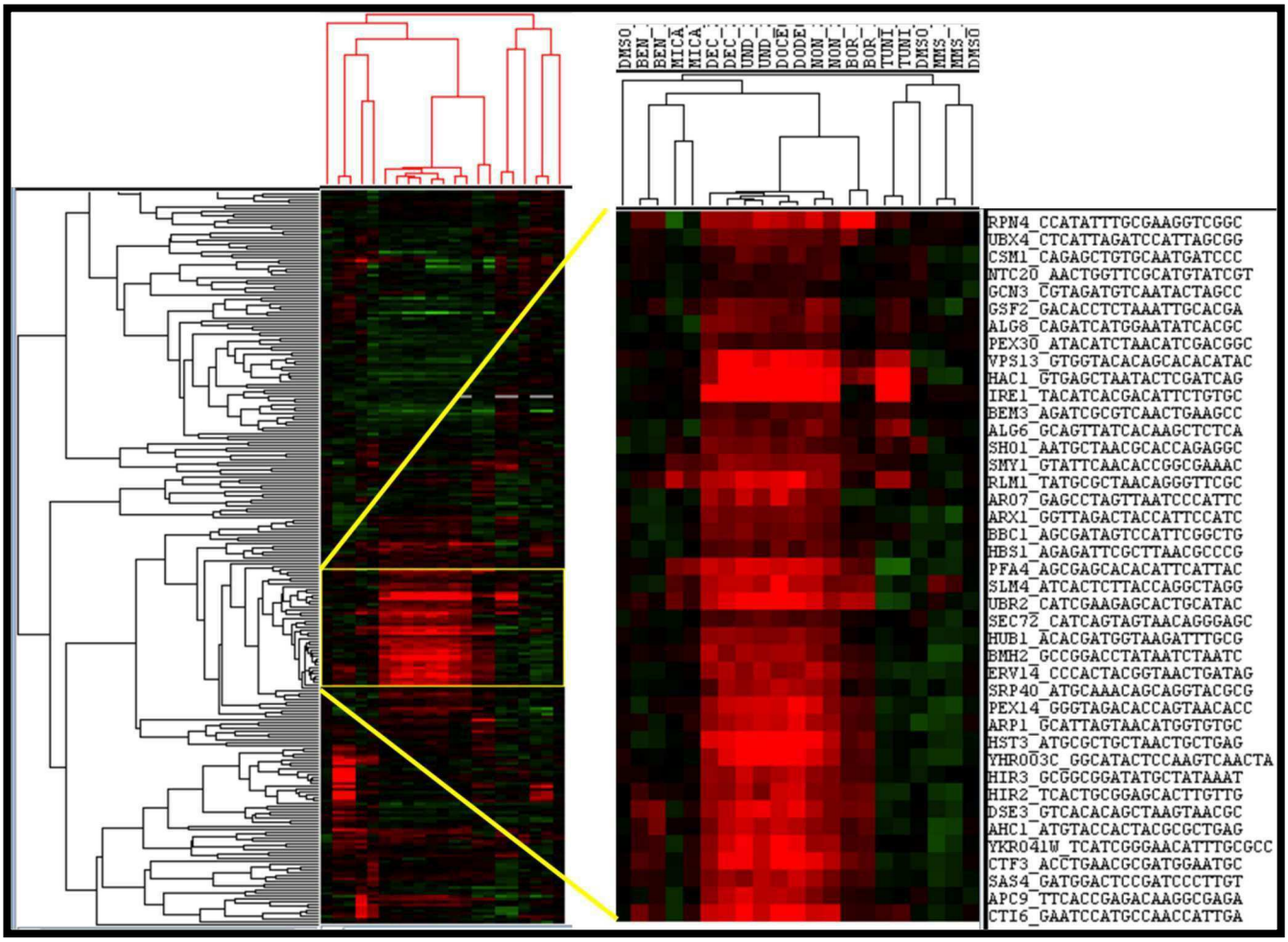


Figura 14 - Gráfico *Java Treeview heatmap*: mutantes coleção *Mini-pool* verso agentes controles, compostos testes e DMSO controle. Vermelho: mutantes hipersensíveis ao tratamento. Verde: mutantes hiper-resistentes ao tratamento. Cinza: sem valor.

Tabela 2 - Correlação dos galatos e os genes possivelmente afetados, acompanhados dos processos envolvidos.

Galatos	**Gene Predito**	**Processos envolvidos**
Nonila	ALG12 LRE1 CUE1	
Decila	CUE1 CSF1 CCC1	- processo de biossíntese do oligossacarídeo ligante a cadeia dolicol - processo catabólico de proteínas associadas ao RE - glicosilação de macromoléculas
undecila	CCC1 ALG12 CUE1	- glicosilação de proteínas - glicosilação - processamento de sinal peptídeo
dodecila	CCC1 RNT1 CUE1	

Tabela 3 - Correlação dos agentes utilizados como controle e os genes possivelmente afetados, acompanhados dos processos envolvidos.

Agente controle	Genes	Processos envolvidos
Tunicamicina (inibidor da glicosilação de proteínas)	SPC3 SPC2 ERV29 SPC3 SPC2 ERV29	- processo de biossíntese do oligossacarídeo ligante a cadeia dolicol - processamento de sinal peptídeo - processo de biossíntese de derivados de carboidrato - glicosilação de macromoléculas e proteínas - processos metabólicos de proteínas
MMS (agente indutor de danos ao DNA)	ELG1 RAD27 TSA1 ELG1 POL3 NSE4	- processos metabólicos/ reparo/ replicação/recombinação/ resposta ao estímulo de danos ao DNA - resposta celular ao estresse/estímulo - processo metabólico de compostos aromáticos/heterocíclicos/cíclicos orgânicos/nitrogenados/macromoléculas celulares - elongação da fita de DNA envolvido na replicação do DNA - processo catabólico de RNA - fase S da mitose do ciclo celular - hidrólise na ligação fosfodiéster de ácidos nucleicos - regulação do ciclo celular
Benomyl (agente despolimerizante de microtúbulo)	TUB3 GIM4 GIM3 TUB3 GIM4	- formação/organização dos complexos: -tubulina, -de proteínas celulares, -de macromoléculas celulares, - enovelamento de proteínas
Bortezoimib (inibidor de proteassoma)	RPT6 SEM1 PBA1 RPT6 IRC25 SEM1	- formação/organização: de partículas regulatórias do proteassoma, do complexo de macromoléculas celulares. - processo catabólico de proteínas proteassomais ubiquitina dependente - regulação positiva ou negativa de sequências específicas de DNA ligados à atividade de fatores de transcrição - regulação positiva da formação/organização do complexo transcricional RNA polimerase II
Micafungica (inibe de forma não competitiva a síntese do 1,3-β-D glucano)	SBE2 ZDS1 KRE2 FKS1 ARC18 PDS1	- biogênese ou organização da parede celular - organização de estruturas de encapsulamento externo - organização da parede celular - regulação do tamanho de componentes celulares - regulação positiva da atividade de quinases e transferases

4.6.4. Método 50:50

O método 50:50 foi utilizado para a obtenção do triplo mutante para os genes de bomba de efluxo PDR1/PDR3/SNQ2, levando a um fenótipo de hipersensibilidade. Dessa maneira a influencia do composto poderia ser analisada sem a interferência desta linha de defesa da célula.

Primeiramente foram obtidos os fragmentos com cerca de 2500 pb, constituídos da marca de seleção URA e regiões homólogas a região do gene a ser deletado (figura 15A). Na primeira transformação, o fragmento foi inserido no genoma da levedura por recombinação. Para a confirmação da inserção nos cinco transformantes (T), foi utilizado o *primer* de confirmação chamado *pop-in* (I). Como controles foram utilizados a cepa selvagem (W) com os *primers pop-in* e *pop-out* (O).

O transformantes positivos apresentaram uma banda com cerca de 950 pb. O controle selvagem testado com o *primer pop-out* apresentou uma banda de 250 pb pois este iniciador tem região homologa a região inserida (região URA) (Figura 15B).

Na segunda transformação, após o plaqueamento do transformante em meio 5-FOA, a região inserida e do gene alvo foram deletados. Para a confirmação da deleção nos 5 transformantes, foi utilizado o *primer* de confirmação *pop-out.* Os transformantes positivos não apresentaram banda. Como controle foi usado o transformante 1, sem sofrer a segunda transformação, e o selvagem para o *primer pop-out* (figura 15C).

A figura 15 é representativa para o processo de deleção de um gene. O mesmo processo foi realizado para os três genes para se obter o triplo mutante.

Após obtenção do triplo mutante, o mesmo foi enviado ao laboratório no Brasil, porém o mesmo ainda não foi utilizado com a sequência GFP para análise das vias afetadas.

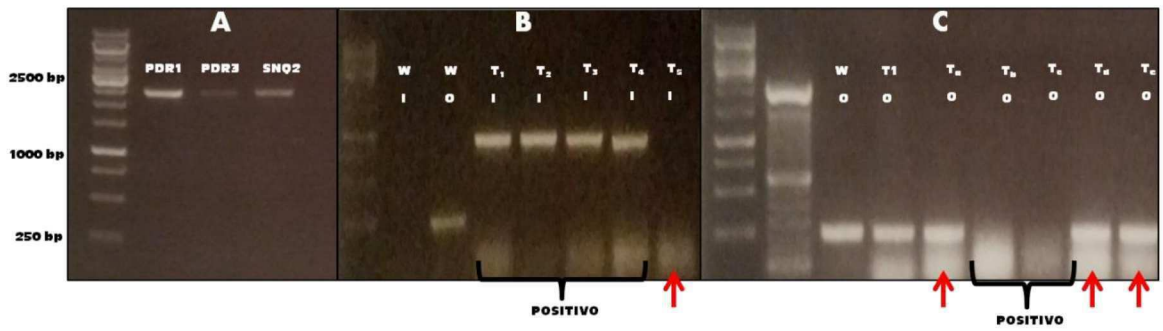


Figura 15 - (A): Produto de PCR: fragmentos PDR1, PDR3 e SNQ2. (B): confirmação para a inserção do fragmento (*pop-in*), positivo: colchete, negativo: seta vermelha. (C): confirmação para a deleção do fragmento inserido no gene alvo (*pop-out*), positivo: colchete, negativo: seta vermelha. Foi utilizado o padrão de 250 a 10000 pb (ThermoFicherScientific-SM1163).

4.7. Cepa UPR-GFP x galatos

A partir de uma coleção GFP com fenótipo não sensível pertencente ao laboratório do Prof. Charles, foi realizada a análise da UPR (*Unfold protein resposnse)*, uma vez que esta via foi afetada com o tratamento com os galatos de acordo com o ensaio de interação genética. Observamos na figura 16 que já após 3 horas de tratamento com os galatos houve aumento na intensidade da fluorescência. Em condições normais está via não esta ativada, ou seja, não é normalmente expressa, porém após o tratamento foi ativada, e o produto de transcrição que contém a sequência GFP aumentou.

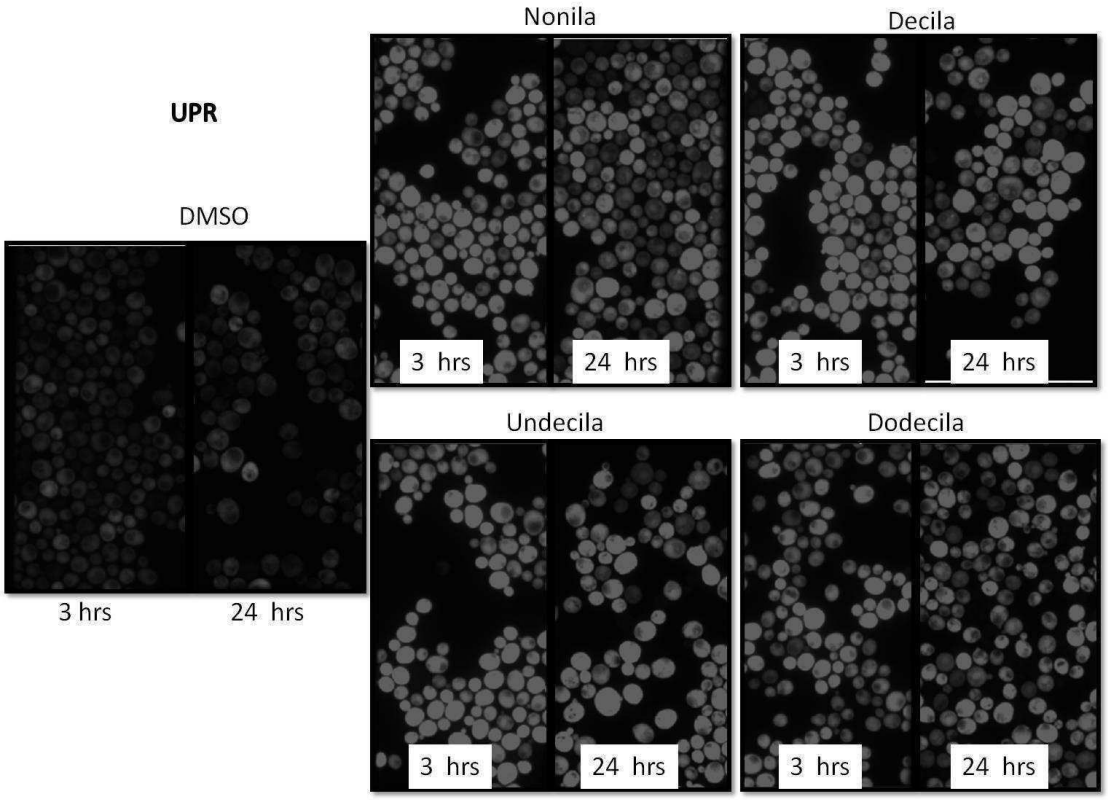


Figura 16 - Análise do estresse ao retículo endoplasmático pelos galatos de nonila, decila, undecila e dodecila. Cepa da levedura *Saccharomyces cerevisiae* com sequencia GFP inserida próxima aos genes do grupo UPR, foi tratada com galatos de nonila, decila, undecila e dodecila na concentração de 22,5 μM por 3 e 24 horas. Por microscópio de fluorescência foi observado a ativação ou não grupo UPR através da fluorescência do grupo GFP expresso concomitantemente.

4.8. Avaliação da n-glicosilação

Uma vez que foram observadas alterações na morfologia do fungo Pl01 após o contato com o G14 e que os galatos apresentaram influencia na via da n-glicosilação ou na resposta de proteínas não enoveladas, foi sugerida a análise de enzimas importantes para o remodelamento e manutenção da parede celular, além de que as mesmas possuem modificações pós traducionais do tipo n-glicosilação, sem as quais perdem as

características funcionais. Assim, as espécies *P. lutzii* (Pl01) e *P. brasiliensis* (Pb18) foram tratadas com o galato de decila, 0,5µg/mL, e posteriormente foram verificadas as atividades enzimáticas da α-(1,3)- glucanase, N-Acetyl-β-D-glucosaminidase e α-amilase. Observou-se que, depois do tratamento para ambas as espécies houve uma redução da porcentagem de atividade em relação ao controle não tratado, em média de 50 % (figura 17).

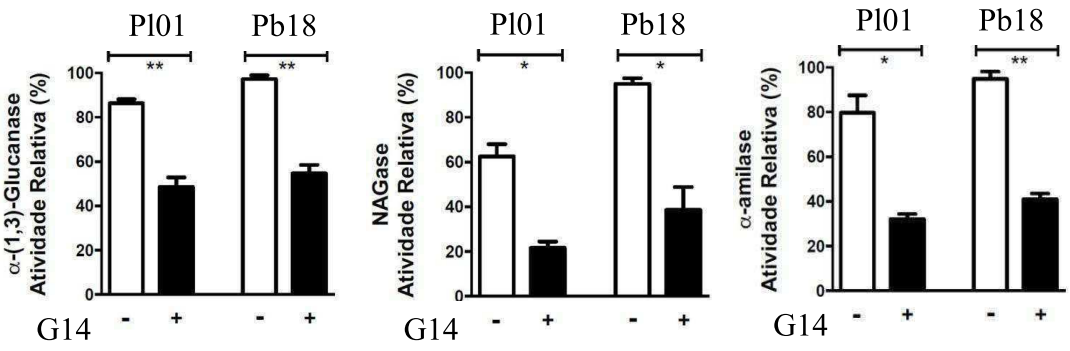


Figura 17 - Análise da influência do galato de decila (G14) na atividade relativa de enzimas n-glicosiladas. Células fúngicas das espécies *P. lutzii* (Pl01) e *P. brasiliensis* (Pb18) foram desafiadas com o G14 na concentração de 0,5µg/mL (+/barra preta) por 72 horas e a porcentagem da atividade relativa das enzimas α-(1,3)- glucanase, N-Acetyl-β-D-glucosaminidase e α-amilase foi avaliada de acordo com o controle sem tratamento (-/barra branca). *p<0,05 em relação ao controle

Uma vez que o processo de n-glicosilação acontece em nivel pós-traducional, sendo um processo essencial para o funcionamento destas enzimas, foi proposto analisar se a queda na atividade da proteína final (enzima) afeta a expressão dos genes relacionados. Concomitantemente, foram analisados os genes IRE1 e HAC1, os quais estão envolvidos na resposta a proteínas não enoveladas (UPR).

A extração de RNA total foi realizada para as espécies *P. lutzii* (Pl01) e *P. brasiliensis* (Pb18) após tratamento com 0,5µg/mL de galato de decila. O RNA total foi quantificado em nanodrop com concentrações acima de 500 ng/µL. Para avaliação de pureza foi observada a relação entre as absorbâncias de A260/280. Esses valores foram observados entre 1,8 e 2,1. Para a confirmação da qualidade do RNA, as amostras foram submetidas à corrida em gel de agarose 1 % e as bandas 18S e 26S do RNAr foram observadas (figura 18).

A figura 19 apresenta a análise da expressão relativa dos genes (NAG, AMY, AGN e AGS) envolvidos na produção das enzimas analisadas anteriormente e genes envolvidos com a reposta ao estresse ao reticulo endoplasmático (IRE1 e HAC1). O gráfico apresenta

quantas vezes os genes estudados estão expressos pela influência do G14, em relação ao controle (sem tratamento).

Para as duas espécies, todos os genes apresentaram aumento da expressão após o tratamento. Em uma análise entre as espécies, observamos que o gene HAC1 apresentou maior expressão na espécie Pb18 (*p<0,05), sendo quase três vezes menos expresso para o Pl01, enquanto IRE1 não apresentou diferença estatística entre as duas espécies. Para os genes AMY, AGN e AGS apresentaram diferença na expressão em relação às duas espécies (p<0,05), enquanto que para o gene NAG não houve diferença estatística. Os genes AMY e AGS foram mais expressos em Pl01, apresentando-se em torno de 4,5 vezes menos expressos para a espécie Pb18. AGN foi mais expresso em Pb18, sendo que para o Pl01 foi quase três vezes menos expresso. Já em uma análise individual, é possível observar um padrão, onde não há diferença estatística entre os genes NAG e AGN, e AMY e AGS, para ambas as espécies.

De modo geral, após o contato com o G14 os genes testados foram hiperexpressos, indicando uma necessidade expressiva do produto destes genes para a manutenção da parede celular fúngica, e ainda ativação dos genes IRE1 e HACA1, o que pode indicar que este evento está culminando no estresse ao reticulo endoplasmático da célula fúngica.

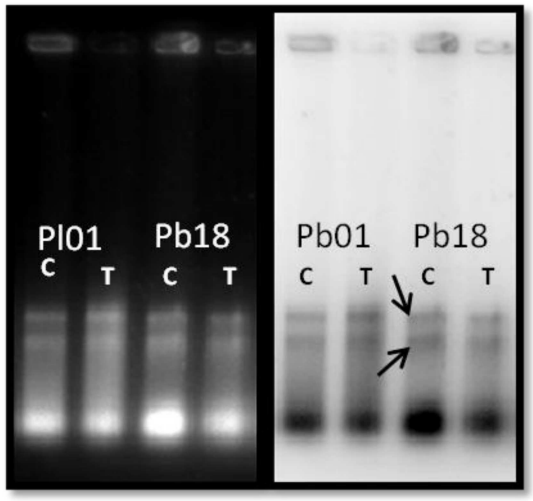


Figura 18 - RNA total submetido a gel de agarose 1 %. Pl01: controle (C) e tratado (T); Pb18: controle (C) e tratado (T). setas: bandas RNAr 26S e 18S, respectivamente.

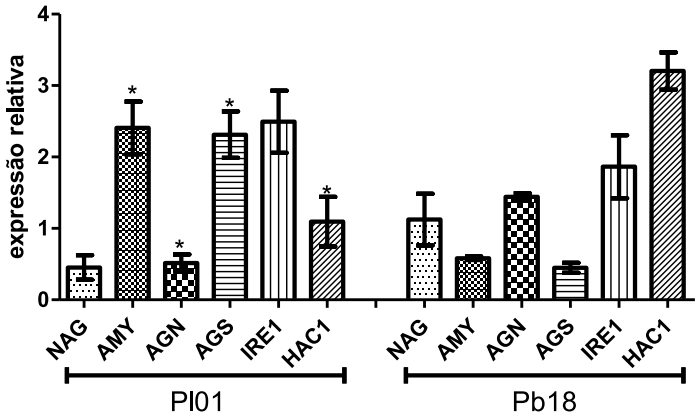


Figura 19 - Análise por PCR em Tempo Real da expressão relativa dos genes que codificam α-(1,3)-glucanase (AGN), α-(1,3)-glucano sintase (AGS), N-Acetyl-β-D-glucosaminidase (NAG), α-amilase (AMY), e os genes sensores de estresse ao retículo endoplasmático IRE1 e HAC, nas células fúngicas das espécies *P. lutzii* (Pl01) *e P. brasiliensis* (Pb18) desafiadas com o galato de decila (G14) na concentração de 0,5µg/mL por 72 horas. Método utilizado para análise: $2^{-\Delta\Delta CT}$. Controle gene endógeno α-tubulina. Controle negativo: amostra substituída por água DEPC. *$p<0,05$ em relação ao gene da outra espécie.

5. DISCUSSÃO

Diferentes antifúngicos (anfotericina B, azóis, e sulfonamidas) podem ser utilizados para o tratamento da PCM. A escolha da terapia dependente da gravidade da doença, do tipo de agente antifúngico disponível e do tempo de uso. Porém em sua maioria, a exigência de prolongados protocolos de administração pode culminar em efeitos tóxicos (BORGES-WALMSLEY et al., 2002; SHIKANAI-YASUDA et al., 2006b; VISBAL et al., 2011). Além disso, nenhuma das terapias envolvidas garante a completa eliminação do fungo no paciente (Travassos e Taborda, 2012a). Neste sentido, é de extrema importância o investimento na descoberta de novos agentes antifúngicos com eficácias superiores e com menos efeitos secundários, a fim de aumentar as opções de tratamento para esta doença.

Assim, a procura por substâncias de origem natural ou seus derivados sintéticos é de extrema importância. O ácido gálico é um destes metabólitos de especial ocorrência no reino vegetal, podendo ser extraído das folhas da espécie *Alchornea glandulosa*, e apresenta diversas atividades biológicas como anti-inflamatória, antimutagênica, anticarcinogênica, antibacteriana, antiviral, analgésica e antioxidante. Como antioxidante, o ácido gálico poderia atuar como agente antiapoptótico e proteger as células humanas contra danos oxidativos (CALVO et al., 2007; TREVIÑO-CUETO et al., 2007; LOPES et al., 2011).

Através de diversos estudos, a molécula de ácido gálico foi sofrendo modificações no intuito de melhorar a sua atividade biológica. Diferentes estudos mostraram que ácido gálico e seus derivados, chamados de galatos de alquila, apresentam atividade antifúngica atuando contra um painel representativo de fungos patogênicos oportunistas (LEAL et al., 2009), bem como já foi estudada a relação estrutura-atividade destas substâncias (KUBO et al., 1995; KUBO et al., 2001). Nosso grupo trabalhou com os galatos de alquila e em estudo anterior foi demonstrado que o derivado do ácido gálico, o galato de decila (G14) apresentou melhor atividade antifúngica *in vitro* contra várias espécies de fungos patogênicos, incluindo *Paracoccidioides* spp. Além disso, este composto mostrou índice de seletividade excelente, o que o tornaria um potencial candidato a protótipo antifúngico de amplo espectro e este resultado incentivou-nos a continuar os estudos para a descoberta de seu mecanismo de ação (DE PAULA; SILVA et al., 2014).

Na literatura foi descrito que o composto galato de nonila provoca danos na membrana celular, porque este composto possui três grupos de hidroxilas hidrofílicas na porção da molécula correspondente ao ácido gálico e uma cadeia alquila hidrofóbica adjacente, perturbando a estrutura estável da bicamada de membrana lipídica. A explicação provável seria que os grupos de hidroxilas interagem com grupos hidrofílicos, empurrando a membrana para formar vínculos de hidrogênio. As cadeias de carbono não polares são dobradas nas bicamadas da membrana, resultando em uma alteração da fluidez desta membrana. Esta lesão provavelmente resulta no vazamento de íons de potássio, além disso, foi observado que este composto pode alcançar o interior da membrana mitocondrial, influenciando as funções mitocondriais relacionadas à produção de espécies reativas de oxigênio (HAWORTH et al., 1993; FUJITA; KUBO, 2002).

Assim, inicialmente neste trabalho foi demonstrado que o composto G14 na concentração de 0,5 μg/mL é capaz de reduzir significativamente a concentração de nitrito no sobrenadante da monocamada da linhagem de macrófagos RAW estimulada por LPS. O mesmo foi realizado por LOPES et al (2005) que demonstraram atividade anti-inflamatória quando macrófagos peritoneais estimulados por LPS foram desafiados com a fração acetato de etila extraída da folha de *A. glandulosa*, apresentando efeito inibitório na produção de óxido nítrico (NO).

Na PCM ocorre uma correlação inversa entre a síntese de TNF-α e a produção de óxido nítrico (GONZALEZ et al., 2000). Nos casos de uma produção exacerbada de óxido nítrico, ocorre um efeito inibitório da imunidade inata e adaptativa do hospedeiro, pois leva a desativação dos macrófagos, reduz a apresentação de antígenos às células dendríticas

pulmonares, inibe a expressão do antígeno pelo MHC-II, controla a produção de citocinas e a expressão de moléculas co-estimuladoras e de adesão, além de aumentar a degradação dos tecidos e diminuir a síntese da matriz extracelular, tendo como consequência a diminuição da eliminação do fungo, formas instáveis do granuloma, e a disseminação do fungo pela via hematogênica (NASCIMENTO et al., 2002; NISHIKAKU et al., 2009; BERNARDINO et al., 2013; BURGER et al., 2013).

Há evidências que o fungo *Paracoccidioides* é capaz de induzir a modulação da resposta inflamatória crônica pela via do óxido nítrico (NISHIKAKU et al., 2009; BERNARDINO et al., 2013), como descrito na literatura que uma lectina ligada a uma porção N-acetilglucosamina foi identificada e denominado paracoccina, a qual foi localizada na superfície da levedura, com capacidade de induzir alta produção de óxido nítrico por macrófagos, influenciando na patogênese da infecção (COLTRI et al., 2006).

Contudo, o óxido nítrico e espécies reativas de oxigênio afetam virtualmente cada etapa do desenvolvimento da inflamação. Os agentes que modulam a atividade de NO, em particular, aqueles que reduzem a formação de NO, podem ter significativo valor terapêutico em processos fisiopatológicos (LOPES et al., 2005). Assim, G14 como outros compostos de origem natural aparentemente teriam efeitos protetores contra o estresse oxidativo em células de mamífero (RONG et al., 1996; LOPES et al., 2005; SCHÄFER; KASCHULA, 2014), atuando como compostos antioxidantes, possibilitando a diminuição do processo inflamatório e os danos gerados.

Na próxima etapa do trabalho foi proposta a análise do perfil de interação do fungo Pl01 com as linhagens pulmonares MRC-5 e A549 e a linhagem de macrófago AMJ2-C11 nos tempos de 1, 3, 6 e 24 horas.

Paracoccidioides spp. é considerado fungo intracelular facultativo, neste sentido possui a habilidade de aderir e invadir as células presentes na barreira do aparato pulmonar. Células epiteliais pulmonares foram demonstradas interagir com este fungo (MENDES-GIANNINI et al., 2004; MONTEIRO DA SILVA et al., 2007; MAZA et al., 2012; ALCANTARA et al., 2015; DE OLIVEIRA et al., 2015; BARROS et al., 2016). Células tipo II cobrem uma pequena área dos alvéolos pulmonares e apresentam distintas funções, como propriedades endocíticas (DEL VECCHIO et al., 2009). Os fibroblastos, como a linhagem MRC-5, são as células mais ubíquas e numerosas dos tecidos de mamíferos. São conhecidas por produzir colágeno e fibronectina, com função principal de reparação, regeneração de tecidos e órgãos danificados, levando a cicatrização dos danos e formação de "fibroses" (RIESKE et al., 2005). Trabalhos como o de Costa et al (2015)

mostram a capacidade de adesão das células leveduriformes de *Paracoccidioides* nas linhagens A549 e MRC-5.

Através da diferenciação das duas populações (fungo e célula) por marcadores específicos de fluorescência, a análise foi realizada utilizando a metodologia de citometria de fluxo pelo equipamento BD FACS Canto IA e citometria por imagens pelo equipamento *In Cell Analyzer 2000*-GE. Nesta análise, as células fúngicas que estão internalizadas não foram distinguidas daquelas somente aderidas. As duas formas de análise são consideradas sensíveis, ou seja, ambas as populações foram bem definidas (marcações específicas de fluorescência e morfologia) e um número acima de 10 mil células foram analisados na presença do fungo, o que confere uma maior acurácia para os resultados. Apesar das células sofrerem influência negativa do fungo, fatores como confluência e morfologia foram monitorados a cada tempo, os quais foram observados satisfatórios para análise da interação (dados não apresentados).

Em ensaios realizados com a linhagem A549, Mendes-Giannini et al (2004) demonstraram que a taxa de interação aumenta progressivamente a partir de 3 horas de contato, e com 5 horas foi possível observar fungos internalizados nas células epiteliais A549. Del Vecchio et al (2009) verificaram que a partir de 5 horas de interação, a taxa diminuiu a cada tempo, uma vez que o fungo já está em fase de internalização. Neste projeto também foi observado um aumento progressivo entre os tempos de 1, 3 e 6 horas, porém com uma taxa menor de interação para a linhagem MRC-5 em relação à linhagem A549. Através de análises proteômicas diferenciais entre as linhagens A549 e MRC-5, foi observado que a linhagem A549 apresenta uma maior expressão de proteínas ligadas ao citoesqueleto e/ou e mobilidade, o que sugere a exposição de um maior número de ligantes/receptores de adesinas expressas pela parede do fungo (RUBPORN et al., 2009).

O ensaio de interação com o macrófago mostrou um perfil de interação diferente das linhagens pulmonares, com aumento da taxa de 3 para 6 horas, e posterior aumento significativo depois de 24 horas de interação. A literatura apresenta trabalhos onde se observou células fúngicas internalizadas com 6 horas de interação (VOLTAN et al., 2013) e outro com células fúngicas internalizadas em macrófagos peritoneais e pulmonares não ativados com 4 horas de interação, além de uma progressiva multiplicação destas células fúngicas em tempos de até 48 horas de interação (BRUMMER et al., 1989).

No tempo de 6 horas foram observadas células internalizadas, e neste tempo foram desafiadas com o composto G14 durante 24 horas. Como na situação sem tratamento houve uma redução na taxa de interação entre os tempo de 6 e 24 horas na linhagem A549,

observou-se importante redução de 1,6 vezes na taxa de interação quando comparado com o tempo de 24 horas, inferindo que o G14 influenciou de certa maneira para que as células fúngicas aderidas e internalizadas fossem eliminadas. Para a linhagem MRC-5 não foi observada redução na taxa de interação depois do tratamento. Para o macrófago AMJ2-C11, com um perfil de aumento da interação entre os tempos de 6 e 24 horas, o composto apresentou redução de 1,2 vezes entre o tempo de 24 horas e o tratamento. A viabilidade das células foi monitorada pelo ensaio de MTT, frente ao composto, indicando que a redução nas taxas de interação não é devida a um efeito negativo (citotóxico) do composto nas células.

Brummer et al (1989) relataram que somente os macrófagos que foram ativados foram capazes de eliminar as células fúngicas internalizadas, impedindo a sua multiplicação no interior do macrófago. Este dado pode inferir que a redução na taxa de interação (entre o tempo de 24 horas de interação e 24 horas de interação mais tratamento) seja pela ação do G14: a) influenciando a célula a ativar com eficiência seus mecanismos de defesa para a eliminação do fungo, ou b) impedindo a adesão de novas células fúngicas à membrana das células estudadas e assim que mais células sejam internalizadas.

No intuito de aprofundar o estudo da atividade antifúngica do G14 em organismos mais complexos, primeiramente foram utilizadas as larvas do modelo *G. mellonella*. Em um trabalho realizado pelo nosso grupo, foi possível padronizar e aplicar a metodologia de citometria de fluxo para este modelo para analisar a interação do fungo com os hemócitos (SCORZONI et al., 2015) nos mesmos tempos propostos para as células. Perfil de interação semelhante foi observado para as linhagens pulmonares. Entre os tempos de 1 e 3 horas, houve um aumento da taxa de interação, e em 6 e 24 horas, redução. Neste modelo a interação de 6 horas quando desafiada pelo G14 por 24 horas não apresentou redução, ou seja, não apresentou diferença estatística em relação ao tempo de 6 horas. Neste caso, pode-se inferir que o G14 não influenciou positivamente para a redução da interação, ou seja, o sistema ficou estabilizado com a mesma taxa por mais 24 horas de incubação.

Tendo em vista esses resultados, uma curva de sobrevivência foi conduzida para avaliar melhor a atividade do composto em um modelo mais complexo. De acordo com a curva, o grupo infectado apresentou uma queda na sobrevivência a partir do sexto dia, como apresentado por Scorzoni et al (2015). O tratamento das larvas com o G14 foi capaz de melhorar a qualidade de vida das larvas até o décimo dia, uma vez que o grupo somente tratado com o composto apresentou alta taxa de sobrevivência até neste dia. Apesar de o grupo infectado apresentar sobrevivência de 50 % no décimo dia, e o grupo

tratado/infectado,75 %, foi observada diferença estatística entre as curvas, o que implica que o G14 tem a capacidade de melhorar a qualidade de vida da larva evitando que não fique debilitada.

Até aqui sugerimos que o G14 influencia positivamente o hospedeiro, modulando a quantidade de óxido nítrico, diminuindo a interação do fungo entre todos os tipos celulares propostos e aumentando o tempo de sobrevivência em *G. mellonella,* além de saber que afeta diretamente o desenvolvimento do fungo.

Além disso, foram também investigados os efeitos do composto sobre a morfologia celular e verificou-se a diminuição da produção de brotamentos, influenciando na proliferação de novas células fúngicas. A formação de brotos é um processo normal para o desenvolvimento e reprodução do fungo, dessa forma o fungo foi desafiado com o G14 na concentração de 0,5 µg/mL. Após 24 horas de contato, pelo ensaio de MTT foi observada diminuição do metabolismo do fungo e ao analisar as células fúngicas foi observada inibição de 38 % na formação de novos brotamentos. Carbonell (1967) relatou que próximo ao local onde o brotamento surge nas células leveduriformes do fungo *Paracoccidioides*, há um acúmulo de mitocôndrias o que indica alta atividade metabólica. Dessa forma, pode-se inferir que o composto tem atividade inibitória a) no metabolismo do fungo e/ou b) na composição e formação da parede celular, sendo que ambos interferem na formação de novos brotamentos. Em estudos realizados com o galato de nonila, foi observada a influência negativa do mesmo sobre a membrana celular fúngica (HAWORTH et al., 1993; FUJITA; KUBO, 2002).

Possível mecanismo de ação do galato de alquila foi descrito em modelo para estudar atividade antibacteriana e observou-se que o composto atuava na estabilidade da membrana, e ainda era capaz de causar alongamento celular e interrupção da divisão celular em *Xanthomonas citri citri*, sugerindo que as proteínas da divisão celular podem ser alvo destes compostos. A divisão celular é um processo potencial que começa com a polimerizaçao de proteínas citoplasmáticas altamente conservadas chamadas FtsZ, formando um anel. Em outros estudos, a FtsZ purificada de *Bacillus subtilis* foi descrita como alvo direto, sendo que os galatos tem a propriedade de perturbar o anel-FtsZ *in vivo*, e causar o alongamento das células. *In vitro*, galatos de alquila ligam-se com elevada afinidade a FtsZ, fazendo com que ao agrupar-se perdem a sua capacidade para polimerizar. As atividades de uma série homóloga de galatos de alquilo com comprimentos de cadeia lateral variando de cinco a oito átomos de carbono foram comparados e galato de heptila apresentou-se como inibidor mais potente da FtsZ (KRÓL et al., 2015).

Fungos patogênicos compartilham as mesmas características da biologia celular com as células dos seus hospedeiros, dessa forma o desenvolvimento de agentes antifúngicos específicos é considerado um desafio. A parede celular é essencial para a manutenção da homeostase osmótica, para formação e manutenção da forma celular e para a morfogênese, além do importante papel na resistência mecânica, o que resulta na proteção da célula contra danos. A parede celular fúngica é retratada como sendo uma bicamada, a qual revela uma camada externa fibrilar que emana a partir de uma camada estrutural subjacente polissacarídica (FREE, 2013).

A parede celular fúngica é considerada única, tornando-se alvo promissor para o desenvolvimento de novos agentes. Entre estes alvos estão os que envolvem a biogênese da parede celular. A parede celular fúngica é composta por polissacarídeos, como as glucanas e a quitina, lipídeos e proteínas, sendo algumas delas altamente glicosiladas (glicoproteínas) (DE GROOT et al., 2005).

Com o objetivo de conhecer o mecanismo de ação deste composto, este projeto propôs também o estudo do G14 por meio de uma coleção de *Saccharomyces cereviseae* com 340 cepas com mutações em genes conhecidos e não essenciais marcadas exclusivamente por *barcodes.* Assim, foi possível fazer um *screening* com o ácido gálico e seus derivados. Com uma curva de dose resposta, a melhor concentração para os galatos de nonila, decila, undecila e dodecila foi selecionada com inibição de crescimento entre 50 e 80 %. A hipersensibilidade dos mutantes em relação aos galatos mostrou que este composto afetou a via da n-glicosilação e enovelamento de proteínas, perfil semelhante encontrado pelo controle Tunicamicina.

Sabemos que algumas proteínas são sintetizadas na porção ribossomal do retículo endoplasmático rugoso (REr) (SHORT; BARR, 2000). No lúmen do REr, a proteína sofre modificações, entre estes o chamado enovelamento proteico adquirindo conformação tridimensional, auxiliadas pelas chaperonas que auxiliam no enovelamento correto, assegurando que estas proteínas não se agrupem ou se enovelem incorretamente, o que resultaria em um processo tóxico para a célula (COOPER, 2000). Logo em seguida, ocorre adição de um precursor denominado oligossacarídeo (glicana), o qual é biossintetizado no citosol do REr. No final esta estrutura é orientada para a face do lúmen do REr e é transferida para a proteína nascente no lúmen do REr por transferases (LODISH et al., 2000). A proteína e o oligossacarídeo são ligados covalentemente na porção n-terminal dos resíduos de asparagina presentes na proteína. Este tipo de processo especificamente é chamado de n-glicosilação, no qual tipicamente a glicoproteína final sempre contém uma

manose e uma porção N-acetilglucosamina (GlcNAc), graças a composição do oligossacarídeo (LODISH et al., 2000). A porção oligossacarídica ou N-glicana tem um importante papel no remodelamento das glicoproteínas, no transporte intracelular e na defesa contra a degradação proteolítica (NAGAI et al., 1997).

No caso de todo este processo sofrer algum tipo de influência negativa, são formadas proteínas não enoveladas, as quais podem se acumular e agrupar, gerando um sinal tóxico para a célula. Em eucariotos, a sinalização deste evento, ou seja, do estresse ao REr, é orquestrada por pelo menos 3 genes: IRE1, ATF6 e PERK. Juntos estes sensores coordenam uma serie de respostas conhecidas como resposta a proteínas não enoveladas (UPR-*unfolding protein response*), a qual é responsável por contornar essa situação e restaurar a homeostasia. Em fungos, nem ATF6 ou PERK foram descritos, mas IRE1é considerado o pilar para manter a homeostasia da célula fúngica. A ativação de IRE1 está intimamente ligada à ativação de HAC1, sendo os dois essenciais para o funcionamento apropriado da resposta a esse estresse (KRISHNAN; ASKEW, 2014).

Neste sentido avaliação de uma cepa GFP, para o sistema UPR frente ao G14, mostrou que este composto é capaz de ativar esta resposta, contribuindo com o resultado predito que ele interfere na n-glicosilação das proteínas, gerando produtos não funcionais e tóxicos para a célula.

Enzimas presentes no espaço da parede celular são responsáveis por unir covalentemente componentes ali presentes, formando uma matriz tridimensional de quitinas, glucanas e glicoproteínas. A perda de algum destes componentes pode prejudicar o crescimento, a morfologia e a viabilidade da célula fúngica (FREE, 2013). *Paracoccidioides* spp, de acordo, com a fase morfológica possui distinta diferenciação na estrutura dos componentes da sua parede celular. Na fase miceliar a parede do fungo é composta majoritariamente por β-1,3-glucana, enquanto que na fase leveduriforme o polissacarídeo α-1,3-glucana está em maior quantidade (Kanetsuna e Carbonell, 1970; Kanetsuna et al., 1972). Este é formado por um simples polímero linear de α-1,3, ligado a um resíduo de glicose e são sintetizadas por glucanas sintases associadas à membrana plasmática, denominadas α-1,3-glucana sintase (AGS1). Esta enzima faz parte de um mecanismo de síntese e hidrolise de α-1,3-glucana, onde a enzima α-1,3-glucanase (AGN1) é capaz de hidrolisar polímeros de glicose ligados por ligações glicosídicas α-1,3, o que facilita o complexo processo de lise, ramificação e retificação de glucanas envolvidos na síntese da parede celular (DE GROOT et al., 2005; VILLALOBOS-DUNO et al., 2013).

A quitina é um polissacarídeo com uma ligação 1,4-β- N-acetil-D-glucosamina (GlcNAc) responsável pela resistência e força da parede celular fúngica. O fungo possui uma maquinaria enzimática que utiliza a quitina externa como fonte de nutriente e ainda para o remodelamento da parede celular durante o ciclo celular. Estas enzimas são classificadas como glicosil hidrolases, as quais podem pertencer a duas grandes categorias: endoquitinases que clivam a quitina aleatoriamente em sítios internos, e as exoquitinases que são divididas em duas subcategorias: quitobiosidases que catalisam diacetilquitobioses e N-acetil-β-d-glucosaminidases (NaG). AS NaGs possuem uma estrutura em forma de barril, a qual na superfície possui um sulco considerado como o domínio catalítico, o qual confere diferentes funções bioquímicas, tais como se ligar a unidades de açúcar presentes na quitina, possivelmente da região (GlcNac), com subsequentemente clivagem da ligação 1,4-β. Em *Paracoccidioides*, a NaG está envolvida na diferenciação e no crescimento do fungo, umas vez que a quitina está presente em maior quantidade na fase leveduriforme (SANTOS et al., 2004; BONFIM et al., 2006; DUO-CHUAN, 2006; KARTHIK et al., 2014). Bonfim et al (2006) identificaram nas quitinases de *Paracoccidioides* domínios glicosiltransferases e α-amilases, sugerindo que este fungo na forma saprofítica (miceliar) recorre a estas enzimas para a aquisição de energia de polissacarídeos presentes no meio.

Na maioria dos fungos, a α-amilase, classificadas como glicosil hidrolases, são secretadas no meio extracelular, atuando na quebra das ligações α-1,4-glicosídicas, envolvidas assim com a degradação do amido e de glicogênio em pequenos oligossacarídeos, os quais podem ser importados para dentro das células e serem utilizados como fonte de energia e carboidrato. Porém alguns estudos indicam que estas enzimas estão associadas à produção e/ou modificação de α-1,3- glucana, ao invés da degradação de amido. As α-glucanas sintases são enzimas localizadas na membrana com dois domínios catalíticos: o C-terminal; domínio intracelular classificado como uma glicosiltransferase, que tem como função a produção de glucana, e o domínio extracelular N-terminal; que possui semelhança à família das amilases e pode estar envolvida com o acoplamento de cadeias de glucano extrudidos (Van Der KAAIJ et al., 2007; CAMACHO et al., 2012; JANEČEK et al., 2014).

A n-glicosilação pode ser alterada por alguns agentes, entre eles o melhor descrito é a Tunicamicina. Esta é classificada como um antibiótico nucleosídeo e inibe a n-glicosilação pelo bloqueio de um N-acetilglucosamina-1-fosfato para um dolicol fosfato presente na membrana na porção do citosol do REr, levando a uma diminuição da glicoproteína final. *P. brasiliensis* quando tratado com o agente Tunicamicina apresentou o

processo de n-glicosilação inibido, assim como uma redução da atividade da α-(1,4)-amilase, o que poderia afetar a composição da parede celular fúngica e desenvolvimento do fungo (DOS REIS ALMEIDA et al., 2014).

Dessa maneira, como foi predito que os galatos afetam a mesma via do agente Tunicamicina, a atividade relativa das enzimas α-(1,3)- glucanase, N-Acetyl-β-D-glucosaminidase e α-amilase foram analisadas por ensaios enzimáticos. Menor atividade enzimática foi observada após o tratamento com o G14, corroborando com os resultados descritos na literatura. A n-glicosilação das proteínas de *P. brasiliensis* é crucial para muitos processos biológicos assim como para interação da levedura com a célula hospedeira, sugerindo que este composto tem potencial para bloquear este processo e interferir na virulência do fungo (DOS REIS ALMEIDA *et al.*, 2011)

No ensaio da expressão relativa dos genes codificantes destas enzimas, foi observado que todos os genes foram hiperexpressos, com relevância significativa em relação ao controle não tratado. Dessa forma a célula fúngica recruta de forma expressiva o produto destes genes, os quais após modificações traducionais seriam enzimas ativas na possível recuperação da parede celular. Porém, com a baixa atividade enzimática destas enzimas, foi pressuposto que o composto galato de decila interfere na via da n-glicosilação, afetando na forma ativa final da enzima e assim na formação adequada da parede celular.

6. CONCLUSÃO

Dessa maneira neste trabalho foi observado que o composto galato de decila (G14) foi capaz de diminuir a quantidade de nitritos no sobrenadante da monocamada da linhagem de macrófagos RAW estimulada por LPS, o que sugere atividade antioxidante.

Através da analise por citometria de fluxo, foi observado que as linhagens pulmonares humanas, MRC-5 e A549, apresentaram o mesmo perfil de interação com *P. lutzii*, entre os tempos estudados. Já a linhagem de macrófago alveolar murino AMJ2-C11 apresentou uma progressiva taxa de interação até o tempo de 24 horas, resultado que difere do perfil dos outros tipos celulares. No modelo mais complexo *G. mellonella* houve uma parcial semelhança com o perfil das linhagens pulmonares humana, confirmando ser um bom modelo para o estudo *Paracoccidioides*-hospedeiro.

Ainda foi possível observar e confirmar a capacidade das células fúngicas de *P. lutzii* em aderir e invadir as linhagens pulmonares MRC-5 e A549 e aos hemócitos da larva de -*G. mellonella* com 6 horas de interação.

Contudo, o composto G14 foi capaz de diminuir a atividade metabólica e a formação de novos brotos das células fúngicas de *P. lutzii*, o que certamente contribuiu para reduzir a taxa de interação de Pl01 a linhagem A549 e aos macrófagos AMJ2-C11, além de apresentar importante capacidade do aumento da taxa de sobrevivência das larvas de *G. mellonella* infectadas com Pl01.

Corroborando com estes resultados, verificamos que o G14 possui forte influência na via da n-glicosilação ou/e da resposta a proteínas não enoveladas, pressupondo seu mecanismo de ação de acordo com a avaliação da interação genética. Esta hipótese ainda foi previamente confirmada pela verificação da baixa atividade enzimática e da contínua expressão dos genes que codificam estas enzimas n-glicosiladas relacionadas à formação da parede celular de *Paracoccidioides* spp.

Assim, o composto G14 é um agente antifúngico capaz de diminuir a capacidade do fungo em reestabelecer a homeostasia, neste caso a reintegração da parede celular, inferindo-se que sua ação antifúngica está ligada diretamente a virulência do fungo, levando a menores taxas de interação fungo-célula hospedeira.

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Capítulo II

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Research Article

Antifungal Activity of Decyl Gallate against Several Species of Pathogenic Fungi

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This work aims to demonstrate that the gallic acid structure modification to the decyl gallate (G14) compound contributed to increase the antifungal activity against several species of pathogenic fungi, mainly, *Candida* spp., *Cryptococcus* spp., *Paracoccidioides* spp., and *Histoplasma capsulatum*, according to standardized microdilution method described by Clinical Laboratory Standard Institute (CLSI) documents. Moreover this compound has a particularly good selectivity index value, which makes it an excellent candidate for broad-spectrum antifungal prototype and encourages the continuation of subsequent studies for the discovery of its mechanism of action.

1. Introduction

In the last two decades, there has been a rapid increase in the incidence of invasive fungal infections (IFIs) caused by fungal pathogens with diminished susceptibility or resistance to many standard antifungal agents. The early treatment of IFIs is essential for optimal clinical outcomes. The effectiveness of standard antifungal drugs (polyenes, azoles, and echinocandins) is not predictable against some emerging fungi and may cause undesirable side effects. Furthermore, the use of antifungals is often inappropriate, exposing patients to adverse effects, drug interactions, and the development of resistance to and super infections by other fungi, reducing their effectiveness and resulting in significant health expenditures. All of these factors are particularly problematic for immunocompromised (IC) or hospitalized patients with serious underlying diseases [1–5]. Considering that the diagnosis of these diseases remains challenging and that treatment is suboptimal, it is difficult to identify and implement the correct therapies.

The use of modified substances from natural compounds as prototypes of molecules for the treatment of diseases has increased abundantly in recent years. However, many of these products have no proven efficacy and safety. The aim of this study was adding new results about the antifungal activity *in vitro* of 14 alkyl gallates against important pathogenic

fungi, mainly, *Candida parapsilosis*, *C. krusei*, *Cryptococcus gattii*, *Histoplasma capsulatum*, and *Paracoccidioides* spp. The literature presents several data about a wide range of biological activities, and its chemical structures have correlation with the antifungal activity and the cytotoxicity [6, 7]. Kubo et al. [8] made an important observation about the length and hydrophobicity of alkyl groups; to a large extent, these factors are associated with their antifungal activity. Because of the wide range of properties and commercial applications, alkyl gallates are compounds of great interest to both pharmaceutical and chemical industries [9]. Besides, we highlighted the decyl gallate role as a promising broad-spectrum antifungal.

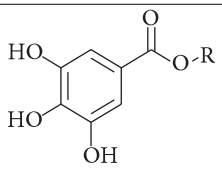
2. Materials and Methods

2.1. Microorganisms. *C. albicans* ATCC 90028 (Ca), *C. krusei* ATCC 6258 (Ck), *C. parapsilosis* ATCC 22019 (Cp), *C. neoformans* ATCC 90012 (Cn), and *C. gattii* ATCC 56990 (Cg) were selected for the study. Four filamentous species were also used, including *T. mentagrophytes* ATCC 11481 (Tm), *T. rubrum* ATCC 28189 (Tr), *A. fumigatus* ATCC 7100 (Af), and *A. niger* ATCC 16404 (An). All species were obtained from the collection of Clinical Mycology Laboratory, School of Pharmaceutical Sciences, UNESP, Araraquara, São Paulo, Brazil. This study also included the dimorphic fungi *H. capsulatum* var. *capsulatum* EH-315 strain (Hc); *P. brasiliensis* isolates 18, D03, and 339 (belonging to the S1 phylogenetic species), isolate 02 (PS2 phylogenetic species), and isolate Epm83 (PS3 phylogenetic species); and *P. lutzii* strain 01 (ATCC MYA-826) and two isolates, EE and 8334MMT (origin is described in Acknowledgments).

2.2. Susceptibility Tests. The minimum inhibitory concentration (MIC; mg L^{-1}) was determined by the antifungal susceptibility test for all species, following the reference broth microdilution method, as outlined in the CLSI. The M27-A3 document [10] was used for yeast and dimorphic fungi species. For dimorphic fungi microdilution test was performed according to de Paula e Silva et al. [11]. The M38-A2 document [12] was used for filamentous species. The determination of the minimum fungicidal concentration (MFC; mg L^{-1}), which is the lowest concentration that did not allow the growth of any fungal colony on the solid medium after the incubation period, was performed as was done by Regasini et al. [13] and Gullo et al. [14]. For this an aliquot from the wells was transferred to a plate with Sabouraud medium (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for the time determined for each species. All the tests were performed in triplicate and in three independents assays. The strain *C. krusei* ATCC 6258 was used also as a quality control for both tests.

The following antifungal drugs were used as controls: amphotericin B (AMB), itraconazole (ITZ), fluconazole (FLZ), terbinafine (TERB), and griseofulvin (GRIS; Sigma-Aldrich, St. Louis, MO, USA). All antifungal drugs were diluted according to the instructions of each CLSI document. The final dilutions and inoculum were in RPMI 1640 medium with L-glutamine without bicarbonate (Gibco; Grand Island, NT, USA) buffered to pH 7.0 with 0.165 M 3-*N*-morpholinopropanesulfonic acid (Sigma-Aldrich, St. Louis, MO, USA) with 2% glucose.

Table 1: Molecular structure of gallic acid (G1) and alkyl gallates (G2–G17).



R	G1	Gallic acid	H
	G2	Methyl gallate	CH_3
	G3	Ethyl gallate	CH_2CH_3
	G4	Propyl gallate	$(CH_2)_2CH_3$
	G5	Isopropyl gallate	$CH(CH_3)_2$
	G6	Butyl gallate	$(CH_2)_3CH_3$
	G7	Pentyl gallate	$(CH_2)_4CH_3$
	G9	Isobutyl gallate	$CH_2CH(CH_3)_2$
	G10	Hexyl gallate	$(CH_2)_5CH_3$
	G11	Heptyl gallate	$(CH_2)_6CH_3$
	G12	Octyl gallate	$(CH_2)_7CH_3$
	G14	Decyl gallate	$(CH_2)_9CH_3$
	G15	Undecyl gallate	$(CH_2)_{10}CH_3$
	G16	Dodecyl gallate	$(CH_2)_{11}CH_3$
	G17	Tetradecyl gallate	$(CH_2)_{13}CH_3$

2.3. Preparation of Alkyl Gallates. Alkyl gallates (Table 1) were synthesized as previously described by Morais et al. [15]. Five milligrams of each dried substance was diluted and solubilized aseptically in appropriate quantities of dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). The amount of DMSO used was previously tested and did not affect the fungal viability (data not shown). For the experiments, the concentration of each substance was calculated for a range of concentrations from 62.5 to 0.002 mg L^{-1} by dilutions in RPMI 1640 medium in a microdilution plate; then the test was performed in accordance with the M27-A3 and M38-A2 documents.

2.4. Cytotoxicity Tests. The cellular cytotoxicity of the alkyl gallates was evaluated against lung tumor cells (A549) and normal fibroblast pulmonary cells (MRC-5), which were obtained from the American Type Culture Collection (Manassas, VA, USA). The cytotoxicity test was performed by MTT [16] using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) at 5 mg mL^{-1}. Spectrophotometric readings were taken from microplates in an ELISA reader (Bio-Rad model 3550) at a wavelength of 540 nm. Untreated cells constituted the positive control (viable cells), and cells treated with hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA) constituted the negative control (death cells). All the tests were performed

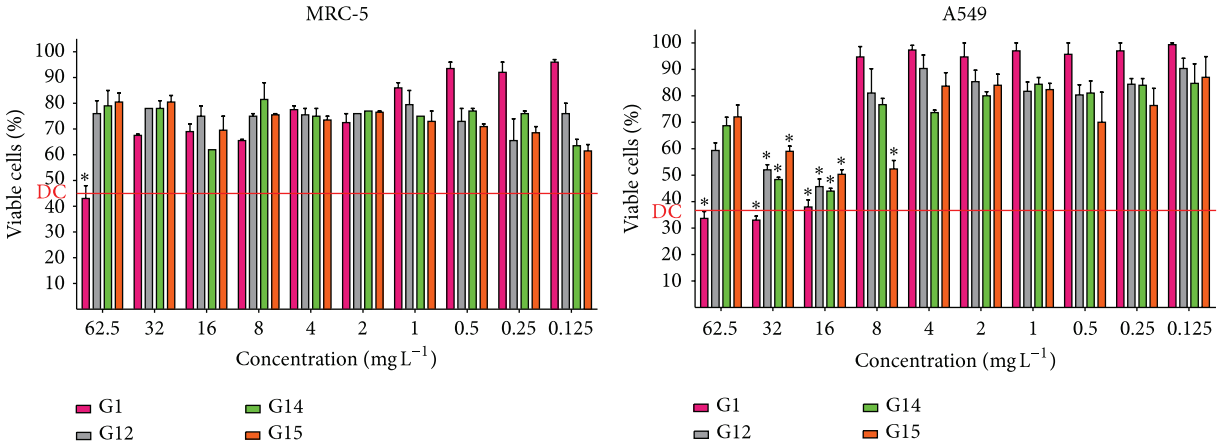


FIGURE 1: Cell viability tested in MRC-5 and A549 after treatment with different concentrations of gallic acid (G1), octyl gallate (G12), decyl gallate (G14), and undecyl gallate (G15). ∗ Indicates that there was no difference statistic ($P > 0.05$) in relation death control (red line represents the mean percentage of viable cells in the death control (DC)).

in triplicate in three independents assays. A test was performed on plates without cells to verify that the reaction cannot occur between alkyl gallates and the reagent to avoid false-positive results (data not shown). Statistical analysis was performed using 2-way ANOVA with Bonferroni post-test using GraphPAd Prism 5 software. P values > 0.05 were considered statistically not significant in relation to the death control.

The IC_{50} values of both cell lines were calculated in relation to G1 molecule and the best antifungal activity of alkyl gallate is the G14. This value represents the concentration required for 50% cell death (i.e., the concentration of each alkyl gallate that results in 50% absorbance reduction compared with untreated cells, termed the IC_{50}) [17]. The selective index (SI) was calculated to both cells lines, which is defined as the ratio of the measured IC_{50} in the two cell lines to the MIC of the tested alkyl gallate (i.e., SI = IC_{50}/MIC). The SI was considered significant when >10 [18, 19].

3. Results

Table 2 shows the MICs of gallic acid (G1) and the alkyl gallates. These molecules correspond to esters with a different number of carbon chains (G2 to G17, as shown in Table 1). Depending on the fungi, the G1 MIC value varied from 4 to 62.5 mg L^{-1} and the same results were observed for G2 to G11. In general, from the alkyl gallates G12 to G15, there was a decrease in the MIC, ranging from 2 to 16 mg L^{-1}, to the majority of fungal species, except to *Aspergillus sp.* and *C. krusei,* which presented value of 31 and >62.5 mg L^{-1}. There was an increase in the MIC value to the alkyl gallates G16 and G17. For the genus *Paracoccidioides*, G12 to G15 presented the lowest MICs for the majority of isolates (0.004 to 0.5 mg L^{-1}), and the G16 showed low MIC values (0.015 to 0.125 mg L^{-1}). The MIC value to *H. capsulatum* was 2 mg L^{-1} for the G12 to G15 alkyl gallates.

Some of the best MICs of the alkyl gallates were similar to or lower than the MICs found for current therapeutic antifungal agents. *C. krusei* and *T. rubrum* are FLZ resistant strains with MIC of 64 mg L^{-1} to these agents, and the G12 and G14 presented MICs of 4–8 mg L^{-1}. Most of the alkyl gallates showed MFC values similar to the MIC values. Nevertheless, G14 had the best MIC when it was evaluated against most fungal species.

The cytotoxicity of gallic acid and the alkyl gallates were *evaluated in respiratory* epithelial cells showing high cell viability to MRC5 and A549 cells line, revealing the low cytotoxicity of these compounds to both cell lines. Figure 1 shows the results of the cytotoxicity test when the cell lines were treated with G1 and the alkyl gallates G12, G14, and G15, that is, those that had the best antifungal activity against most fungi species tested in this study, and we could observe that these selected alkyl gallates showed low cytotoxicity to both cell lines. In this test, the viability percentage is considered satisfactory when it is above the mean percentage of death control. In Supplementary Material we present the results of the cytotoxicity test to all alkyl gallates used in this study (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/506273).

Table 3 presents the IC_{50} values of A549 and MRC-5 cell lines for G1 and G14. These alkyl gallates showed IC_{50} values above the MICs and MFCs determined for the different fungi. The SI was also demonstrated in Table 3, and we could observe that G14 showed the greatest antifungal activity against the majority of fungal species and had higher SI values for both the MRC-5 and A549 cell lines.

4. Discussion

Gallic acid (GA) or 3,4,5-trihydroxybenzoic acid is a natural plant triphenol and it can be produced by acid hydrolysis of tannic acid. The substitution of the GA acid portion allows the obtainment of analogues esters with distinct physicochemical characteristics, especially lipophilicity that is evaluated by

Table 2: MICs/MFCs of gallic acid and alkyl gallates against different fungal species.

	Ca	Ck	Cp	Cn	Cg	Tm	Tr	Afu	Ani	Hc	EE	8334MMT	01	18	D03	339	02	Emp83
G1	62.5/#	31/#	31/#	31/>62.5	31/>62.5	31/#	16/#	>62.5/#	>62.5/#	1/#	8/#	>62.5/#	4/#	16/#	>62.5/#	31/#	4/#	4/#
G2	>62.5/#	>62.5/#	>62.5/#	>62.5/#	16/>62.5	62.5/#	62.5/#	>62.5/#	>62.5/#	31/#	4/#	16/#	2/#	16/#	16/#	16/#	16/#	1/#
G3	>62.5/#	>62.5/#	>62.5/#	>62.5/#	16/>62.5	62.5/#	62.5/#	>62.5/#	>62.5/#	62.5/#	8/#	16/#	1/#	8/#	4/#	8/#	16/#	1/#
G4	>62.5/#	>62.5/#	>62.5/#	>62.5/#	16/>62.5	62.5/#	31/#	>62.5/#	>62.5/#	31/#	2/#	8/#	1/#	8/#	2/#	4/#	4/#	0.25/#
G5	>62.5/#	>62.5/#	>62.5/#	62.5/>62.5	16/>62.5	62.5/#	31/#	>62.5/#	>62.5/#	16/#	2/#	4/#	2/#	16/#	2/#	4/#	4/#	0.015/#
G6	>62.5/#	>62.5/#	>62.5/#	16/>62.5	8/>62.5	31/#	16/#	>62.5/#	>62.5/#	16/#	2/#	8/#	2/#	2/#	2/#	4/#	4/#	0.015/#
G7	>62.5/#	>62.5/#	>62.5/#	8/>62.5	4/>62.5	16/#	16/#	>62.5/#	>62.5/#	4/#	0.5/#	2/#	0.25/#	0.5/#	0.25/#	1/#	1/#	0.015/#
G9	>62.5/#	>62.5/#	>62.5/#	16/>62.5	4/>62.5	31/#	31/#	*	*	8/#	4/#	8/#	1/#	2/#	1/#	4/#	2/#	0.25/#
G10	62.5/#	31/>62.5	31/#	8/31	2/31	16/#	8/#	>62.5/#	>62.5/#	4/#	0.5/#	2/#	0.25/#	0.25/#	0.25/#	0.5/#	2/#	1/#
G11	16/31	31/#	31/#	2/16	1/16	4/#	8/#	62.5/#	31/#	2/#	0.125/#	0.25/#	0.125/#	0.125/#	0.125/#	0.03/#	0.5/#	0.25/#
G12	8/#	8/#	8/#	2/8	1/8	8/#	8/#	31/#	31/#	2/#	0.125/#	0.25/#	0.125/#	0.015/#	0.125/#	0.015/#	0.25/#	0.015/#
G14	4/#	4/31	4/#	1/4	1/2	4/#	8/#	31/#	8/#	2/#	0.03/#	0.125/#	0.125/#	0.004/#	0.125/#	0.004/#	0.004/#	0.008/#
G15	2/#	4/>62.5	2/4	1/4	0.5/4	4/#	4/#	>62.5/#	>62.5/#	2/#	0.03/#	0.03/#	0.125/#	0.015/#	0.125/#	0.008/#	0.03/#	0.015/#
G16	2/62.5	4/>62.5	4/16	1/4	1/4	4/#	4/#	>62.5/#	>62.5/#	4/#	0.03/#	0.03/#	0.125/#	0.125/#	0.125/#	0.06/#	0.015/#	0.5/#
G17	4/>62.5	4/>62.5	31/#	1/16	0.5/4	4/#	>62.5/#	>62.5/#	>62.5/#	>62.5/#	0.125/#	0.25/#	0.125/#	0.25/#	0.125/#	0.125/#	0.03/#	0.06/#
AMB	**1**	**2**	**0.5**	**0.25**	**0.5**	*	*	**8**	**4**	**0.03**	**0.25**	**0.25**	**0.06**	**0.125**	**0.008**	**0.06**	**0.125**	**0.06**
ITZ	**0.5**	**0.5**	**0.25**	*	*	*	*	**>16**	**>16**	**0.03**	**0.008**	**0.03**	**0.008**	**0.015**	**0.015**	**0.015**	**0.008**	**0.015**
FLZ	**1**	**64**	**2**	**4**	**4**	**1**	**64**	*	*	**0.125**	*	*	*	*	*	*	*	*
TERB	*	*	*	*	*	**0.008**	**0.03**	*	*	*	*	*	*	*	*	*	*	*
GRIS	*	*	*	*	*	**0.5**	**1**	*	*	*	*	*	*	*	*	*	*	*

#MFC = MIC, *compound not tested for this species.

Table 3: The IC_{50} and SI values for both cell lines and all fungi species against gallic acid (G1) and decyl gallate (G14).

	SI			
	G1		G14	
	MRC-5	A549	MRC-5	A549
Ca	0	0	>10	2
Ck	0	0	2	>10
Cp	0	0	>10	>10
Cn	0	0	>10	>10
Cg	0	0	>10	>10
Tm	0	0	>10	>10
Tr	0	0	6	9
Af	0	0	2	2
An	0	0	6	9
Hc	3	2	>10	>10
EE	0	0	>10	>10
8334MMT	0	0	>10	>10
1	1	1	>10	>10
18	0	0	>10	>10
D03	0	0	>10	>10
339	0	0	>10	>10
2	1	1	>10	>10
Epm83	1	1	>10	>10
IC_{50} (mg L^{-1})	73	93	50	71

the partition coefficient, called alkyl gallates, according to the atom carbon number in the side chain [6, 7]. GA acts as an antiapoptotic agent and protects human cells against oxidative damage, as it has the ability to scavenge and reduce reactive oxygen species (ROS) formation [20]. The alkyl gallates, like methyl, propyl, octyl, and dodecyl gallates, have a wide range of biological activities, used in food manufacturing as antioxidants, as well as in the pharmaceutical and cosmetic industries, as well as antifungal [6, 21], antibacterial [22], antiviral [23], antitumoral [7], and antihemolytic activities [24]. Because of these several interesting properties and commercial applications, alkyl gallates are compounds of great interest to both pharmaceutical and chemical industries.

Our group showed MIC values equivalent to the values found by Leal et al. [6], which confirm the reproducibility of antifungal activity of the alkyl gallates, and brings new results with other important pathogenic fungi, as *C. parapsilosis*, *C. krusei*, *C. gattii*, *H. capsulatum*, and *Paracoccidioides* spp. Current increases of antifungal drug resistance in *Candida* spp. and clinical treatment failures are of concern, as invasive candidiasis is a significant cause of mortality in intensive care units [25]. Cryptococcosis is an important globally infectious disease. The majority of illness is among patients with defective cell-mediated immunity. The most common clinical presentation is *Cryptococci* meningitis, with over 1 million cases and 600,000 deaths per year [26]. Between the most commonly endemic mycoses described are paracoccidioidomycosis and histoplasmosis; both are difficult to diagnose, because of limiting factors as isolation conditions and sensitivity and specificity of microscopic examination of fluids and tissues which does not lead to immediate diagnosis, hindering successful subsequent treatments [27]. These difficulties constitute serious problems and underscore the need for a better understanding of the pathophysiology of different fungal infections, which could help to identify new targets of drug therapy and lead to the development of new antifungal agents [2, 3].

Then, to evaluate the susceptibility to gallic acid and alkyl gallates, 18 fungi species were assayed. G1 showed high MIC value for most isolates and the same results were observed for G2 to G11, suggesting that the esterification process in the original G1 molecule did not improve the antifungal activity. However, the esterification process for the formation of G12 to G15 showed MICs values significantly different; in other words, chains with eight to eleven carbons enhanced the antifungal activity. These findings indicate that this modification contributed positively to improvement of the antifungal activity of the compound. However, this pattern was not observed in G16 and G17 against most isolates. Instead, higher MICs values were recorded, suggesting a cutoff limit for the chain size.

There was not a change in the susceptibility among the alkyl gallates that presented antifungal activity and the species of same genus, but the MIC value found was considered low. This pattern was observed for *Candida* and *Cryptococcus* species, important yeasts more frequently in the last years in critically ill patients [25, 26]. These results confirm the hypothesis that these compounds can be better studied to find the unique compound against the majority of clinical importance fungi.

Histoplasma capsulatum showed constant MIC values to G12 to G15, which indicate that the chains with eight to eleven carbons have the same effect on this fungus, and the *Paracoccidioides* genus is more susceptible to alkyl gallates than the other fungal species tested. These results show that these compounds can be effective in cases of therapies with difficult diagnosis of specific species or genus. High susceptibility between the species *P. lutzii* and *P. brasiliensis* was observed suggesting the necessity of more studies about the mechanism action involved.

We agree with the conclusion presented by Leal et al. [6] that the activity can vary with the fungi tested, mainly between groups of fungi, and that the antifungal activity of alkyl gallates appears to be dependent on the presence of a catechol moiety along with a hydrophobic alkyl chain, similar to the activity of alkanols described by Kubo et al. [28].

Several studies suggest the mechanism of action involves these compounds. Fujita and Kubo [29] suggested by glucose-induced medium acidification method [30] that an alkyl gallate with nine carbons in the chain side causes damage in the cellular membrane including the plasma membrane, because this compound has three hydrophilic hydroxyl groups in the head of gallate acids and the hydrophobic alkyl chain in the tail, disturbing the stable structure of lipid membrane bilayers. The probable explanation is that hydroxyl groups would interact with hydrophilic groups thrusting on the membrane to form hydrogen binds. The nonpolar carbon chains are folded into the membrane bilayers, resulting in

a change of the membrane fluidity. This injury probably resulted in the leakage of potassium ions from fungi cells. This study also suggested that this alkyl gallate might achieve inner membrane of mitochondria, influencing mitochondrial functions related to ROS generation.

The development of an antifungal agent is considered challenging because potential targets can be shared by yeast and mammalian cells; both are eukaryotes and have some homologous metabolic pathways. The optimal antifungal agent should have a wide activity spectrum, have a fungicidal action rather than fungistatic action, be available for oral and parenteral use, be safe in the efficacious dose, have a great cost-effectiveness, and be stable to microbial resistance. Considering all these points, novel and selective molecular targets for the development of new antifungal agents with the goal of minimizing toxicity are of great importance.

In this sense, *in vitro* cytotoxicity assays are the first step to assess whether a compound with potential antifungal activity is promising to become a future antifungal agent. In this study, two lung cell lines were used for the cytotoxicity by MTT assay, since the majority of fungi tested are bound to the respiratory tract. Then, the cell viability for both lines remained greater than 50% at most of concentrations of G14 and the other two alkyl gallates, G12 and G15, considered with good antifungal activity, by the MTT assay.

MTT assay detects the decrease of viable cells number by the reduction of tetrazolium salt to formazan into living cells that occurs mainly through electron transfer at the mitochondrial level [16, 31]. There are in the literature several reports about the cytotoxicity of alkyl gallates. Locatelli et al. [7] described in a review that alkyl gallates are not cytotoxic against rat liver slices and/or nontumoral cell line (monkey kidney fibroblasts, VERO cells), normal mouse brain endothelial cells, human lymphocytes, or when administered in rats or mice.

Another factor to consider is the detachable selectivity index. The SI is an indication of the relative safety of a compound, where higher SI values reflect greater safety, once that this value is defined as the ratio of the IC_{50} for the MIC value. In other words, the necessary concentration of the alkyl gallate to kill 50% of the health mammalians cells is higher than the concentration considered fungicide. Then SI will be greater than 10 because the values are inversely related. The ratio between the safety and potency of a compound is a very important parameter to consider in the development of therapeutic products [17, 32, 33]. Accordingly, the alkyl gallate G14 had better MIC values and presented an SI above 10 against most fungi, revealing that this alkyl gallate is safe. These data suggest the importance of more studies about the difference of action mechanism between the fungal and mammalian cells, searching for the explanation of the fact that an alkyl gallate with a ten-carbon chain had better activity than the others, with one carbon to more or less.

A point that should be emphasized in favor of this compound is that most of MIC values were similar to or lower than the values of standard antifungal agents, indicating the importance of these substances as promising antifungal agents. One of the major obstacles to antifungal therapy is toxicity, the associated high cost, the development of intrinsic resistance, or a reduced susceptibility to available antifungal agents.

Amphotericin B is considered the "gold standard" to mainstay of antifungal therapy because of its broad-spectrum activity and few cases of mycological resistance. However, it is associated with frequent and potentially serious adverse effects. The serious adverse effects led to the development of AmB formulations, which presented lower rates of nephrotoxicity, but the cost of these agents is substantial, and access is limited in resource-limited settings [34, 35]. On the other hand, the recent increases in *Candida* spp. resistance to echinocandins and azoles have led to clinical failures. This is a matter of concern because of the limited number of drug classes targeting different fungal components and because the number of patients at risk receiving treatment is continually growing, thus further increasing antifungal drug pressure [25]. In this sense, besides of the possibility of a treatment isolate with this alkyl gallate, a combinatory therapy of the alkyl gallate with the available antifungals agents can be an option that can lead to reduce the dose or improving the action of both antifungal agents.

In conclusion, in this preliminary study, among a series of 14 alkyl gallates, our group selected the decyl gallate that was considered to have the best antifungal activity for a wide variety of pathogenic fungi with clinical importance. These suggest how important the structure activity relationship is to obtain the best antifungal performance. It is a promising compound for further studies once the cytotoxicity tests were compatible with those already described in the literature, and the fungicide action mechanism is not prejudice to mammals cells, showing a SI that guarantees the safety required in this initial step.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Importance of adhesins in virulence of *Paracoccidioides* spp.

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Members of the *Paracoccidioides* genus are the etiologic agents of paracoccidioidomycosis (PCM). This genus is composed of two species: *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. The correct molecular taxonomic classification of these fungi has created new opportunities for studying and understanding their relationships with their hosts. *Paracoccidioides* spp. have features that permit their growth under adverse conditions, enable them to adhere to and invade host tissues and may contribute to disease development. Cell wall proteins called adhesins facilitate adhesion and are capable of mediating fungi-host interactions during infection. This study aimed to evaluate the adhesion profile of two species of the genus *Paracoccidioides*, to analyze the expression of adhesin-encoding genes by real-time PCR and to relate these results to the virulence of the species, as assessed using a survival curve in mice and in *Galleria mellonella* after blocking the adhesins. A high level of heterogeneity was observed in adhesion and adhesin expression, showing that the 14-3-3 and enolase molecules are the most highly expressed adhesins during pathogen-host interaction. Additionally, a survival curve revealed a correlation between the adhesion rate and survival, with *P. brasiliensis* showing higher adhesion and adhesin expression levels and greater virulence when compared with *P. lutzii*. After blocking 14-3-3 and enolase adhesins, we observed modifications in the virulence of these two species, revealing the importance of these molecules during the pathogenesis of members of the *Paracoccidioides* genus. These results revealed new insights into the host-pathogen interaction of this genus and may enhance our understanding of different isolates that could be useful for the treatment of this mycosis.

Keywords: ***Paracoccidioides*** **spp., virulence, adhesion, adhesins**

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Introduction

Members of *Paracoccidioides* spp. are dimorphic fungi and the etiological agents of paracoccidioidomycosis (PCM), a systemic mycosis affecting people in Latin America. Brazil has the largest number of endemic PCM areas in the world (Franco et al., 1993).

Matute et al. (2006) classified the *Paracoccidioides* genus into three phylogenetic species: S1, PS2, and PS3. These species have different geographic distributions: S1 is a paraphyletic group found in Brazil, Argentina, Paraguay, Peru, and Venezuela; PS2 is a monophyletic group found in Brazil and Venezuela; and PS3 is a monophyletic group found only in Colombia. Carrero et al. (2008)

classified these isolates into two phylogenetic species, S1 and PS3, which were previously described by Matute et al. (2006), and also classified isolate Pb01 as a new phylogenetic species. Teixeira et al. (2009a) proposed that the Pb01 isolate was a new species, *P. lutzii*. The newly revised molecular taxonomy for these fungi has created new opportunities for studying and understanding their eco-epidemiological relationships with their hosts (Bagagli et al., 2006; Teixeira et al., 2009a). *P. lutzii* and *P. brasiliensis* are found in the west-central and in the southern/southeastern regions of Brazil, respectively (Gegembauer et al., 2014).

The agents of systemic mycoses have some properties that permit their growth in the adverse conditions of the host and may contribute to the development of disease (Casadevall and Pirofski, 1999). *Paracoccidioides* spp. have mechanisms that enable them to adhere to and invade barriers imposed by the host tissues (Mendes-Giannini et al., 1994, 2006). The fungi synthesize several substances that participate directly or indirectly in the parasite-host relationship (Mendes-Giannini et al., 2000). Therefore, the successful colonization of the host tissues by the fungus is a complex event, usually involving a pathogen ligand and a host cell receptor. The microorganism has three host components with which it can interact: products secreted by the cell, surfaces of the host cell, and proteins of the extracellular matrix (ECM), such as type I and IV collagens, fibronectin and laminin (Mendes-Giannini et al., 2006). The understanding and identification of molecules involved in the adhesion of microorganisms to different substrates in the host can aid in the discovery of efficient treatments for systemic mycoses.

Paracoccidioides virulence is a multifaceted event involving the expression of multiple genes at different stages of infection (da Silva et al., 2013). Several adhesins have been described in *Paracoccidioides* spp. and these characterizations have most often been performed using the Pb18 and Pb01 strains. The importance of the following adhesins in fungus-host interactions has been examined: GP43 (Hanna et al., 2000; Mendes-Giannini et al., 2006), 14-3-3 protein (30 kDa) (Andreotti et al., 2005), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Barbosa et al., 2006), triosephosphate isomerase (TPI) (Pereira et al., 2007), enolase (Donofrio et al., 2009; Nogueira et al., 2010; Marcos et al., 2012), and malate synthase (da Silva Neto et al., 2009) using different methodologies like ligand affinity binding, inhibition using antibodies, biding, biding competition, Far-Western blot, ELISA, and Western blot assays.

Besides its direct participation in adhesion, most of the described adhesins for *Paracoccidioides* spp. are enzymes that participate in the glycolytic pathway, tricarboxylic acid cycle and glyoxylate cycle in Paracoccidioides spp. acting as "moonlighting proteins" (Marcos et al., 2014). Recent studies have shown the presence of these enzymes in extracellular vesicles produced by the fungi as well as being present in the cell wall, wherein blocking them leads to a decrease in the ability of the fungi to adhere to cellular host components (Puccia et al., 2011; Vallejo et al., 2011, 2012a,b).

The presence of these enzymes at the yeast cell surface in the absence of a conventional N-terminal signal sequence responsible for targeting the protein into the classical secretory pathway is an intriguing question in the study of the paracoccidioimycosis. Some of these enzymes could behave as anchorless adhesins, which bind to the cell wall and allows direct interaction with the host (da Silva Neto et al., 2009). However, its known that in the N-terminal half of the *C. albicans* GAPDH polypeptide encoded by the TDH3 gene, for example, is able to direct its incorporation into the yeast cell wall (Delgado et al., 2003) but, in the case of the fungi from the *Paracoccidioides* genus, more studies are needed to identify putative signals related to its cell wall targeting.

How they interact with the host cells is another intriguing question. Marcos et al. (2012) demonstrated that the *P. brasiliensis* enolase has a plasminogen-binding motif (RGD) ($_{254}$FYKADEKKY$_{262}$), that is involved in the direct interaction with the host cell surface. It was shown, that when the RGD motif was added as a competitor in the binding assay of enolase to pneumocytes, the binding of the enzyme to the host cells decreased 10%, demonstrating the role of this motif in the enzyme binding to cellular components of the host, however more studies should be done to better understand how these adhesins interacts with the host.

The virulence of fungi may be evaluated using murine models. More recently, the larvae of *G. mellonella* are increasingly being used as an infection model for studying the virulence factors and pathogenesis of many fungal and bacterial pathogens (Cook and McArthur, 2013). Murine models are still considered the gold standard for the study of pathogenesis; however, economic, logistical and ethical considerations limit the use of mammalian host models of infection, especially when the experiment requires the analysis of a large number of strains (Jacobsen, 2014). Thomaz et al. (2013) used *G. mellonella* to evaluate the efficacy of this model for the study of the dimorphic fungi *P. lutzii* and *Histoplasma capsulatum* and concluded that this is a potentially useful model for studying the virulence of dimorphic fungi.

Thus, this study aims to investigate adhesins in the two species of the *Paracoccidioides* genus and to understand how the capacity of these species to express adhesins affects their virulence.

Materials and Methods

Ethics Statements

Animal experiments were performed according to Brazilian Federal Law 11.794, which has established procedures for the use of animals in scientific research, and to state law, which has established the code of animal protection of the State of São Paulo. For this study, male BALB/C and C57BL/6 mice (3–4 weeks old) were used, and all efforts were made to minimize the suffering of the animals. The experiments were approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences of Araraquara—UNESP FCFAr (Case 10/2011/CEUA/FCF).

Microorganisms

Experiments were performed with two isolates belonging to two species of the genus *Paracoccidioides*: *P. brasiliensis* (Pb18) and *P. lutzii* (Pb01). Pb18 is representative of the major phylogenetic group S1 and has been extensively used in the literature due to its demonstrated virulence in mice when inoculated by the intraperitoneal, intratracheal and intravenous routes

(Calich et al., 1998). Pb01 is a clinical isolate from an acute form of PCM in an adult male and is the most thoroughly studied isolate at the molecular level, as shown by an extensive analysis of its transcriptome that yielded ESTs covering approximately 80% of the estimated genome of *P. lutzii* (Felipe et al., 2005b). These isolates were maintained in the mycelial phase (M) and were transferred to Fava-Netto medium supplemented with fetal bovine serum to revert to the yeast phase (L) before the experiments. After reversion, isolates were maintained in Fava-Netto medium without any supplements. Consecutive subcultures were generated each week. The isolates were used for experiments after the 4th subculture. Before the experiments, the fungi were growth for 4 days in brain heart infusion medium (BHI) supplemented with glucose (2%) at 37°C with shaking at 250 rpm.

Adhesion to A549 Pneumocytes of *P. brasiliensis* and *P. lutzii*

Cells from the A549 epithelial lineage (type II pneumocytes from the Rio de Janeiro Cell Bank) were grown in 6-well cell plates in HAM F-12 medium supplemented with 10% fetal bovine serum. After monolayer formation, the cells were washed three times with sterile phosphate-buffered saline (PBS) and inoculated with a *Paracoccidioides* spp. inoculum of 5×10^6 cells/mL. The standard inoculum of *Paracoccidioides* spp. in PBS had previously been incubated for 15 min at 37°C with 10 μM CFSE [5(6)-carboxyfluorescein diacetate N-succinimidyl ester], which stains the fungal cell wall, washed and resuspended in PBS. The infected cells were then incubated for 2–5 h at 36.5°C and 5% CO_2. After incubation, the cells were removed from the plates using 0.2% ATV-trypsin solution and 0.02% Versene (Adolfo Lutz). The trypsinized cells were washed in medium containing fetal bovine serum, centrifuged and fixed with 4% paraformaldehyde. The fixed cells were then evaluated using flow cytometry (FACSCanto, BD) to determine the amount of adherent fungi and to thereby determine the infective capacity of the different species of the *Paracoccidioides* genus. Experiments were performed in triplicate with three independent experiments for each species. Statistical analyses were performed using One-Way ANOVA with Tukey's coefficient. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).

Adhesion of *P. brasiliensis* and *P. lutzii* to the Extracellular Matrix Components Laminin, Fibronectin and Type I and Type IV Collagen

Twenty-four-well plates (Corning®) were coated with the ECM components laminin, fibronectin and type I and IV collagen (Sigma Aldrich) at 50 μg/ml for 18 h at 4°C and 1 h at room temperature. After the sensitization period, the plates were washed three times with PBS. For the adhesion assay, according to Oliveira et al. (2014), inocula of different phylogenetic species of *Paracoccidioides* (5×10^6 cells/mL) were prepared in PBS. Then, 500 μL of these inocula were added to the plate, which had already been coated with components of the ECM, as described above. The plates were incubated at 37°C for 2 h. After the incubation, the supernatant was removed, and the plates were washed three times with PBS. To remove the fungus that adhered to the different ECM components, 300 μL of 0.2% ATV-trypsin solution and 0.02% Versene (Adolfo Lutz) was added to each well. Trypsin suspensions were collected and centrifuged at 2500 rpm at 4°C. Subsequently, the supernatants were discarded, and 500 μL of FACSFlow was added for subsequent cell counting by flow cytometry (FACSCanto, BD) using the BD FACSDiva software (BD). These experiments were also performed in triplicate, with three independent experiments for each species. Statistical analyses were performed using one-way ANOVA with Tukey's coefficient. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).

Expression Analysis of the Genes Encoding Adhesins by Real-Time PCR

The expression of genes encoding seven adhesins, which were previously described in *Paracoccidioides* spp., was evaluated in the different species. Specific primers for each gene were synthesized (**Table 1**). The L34 gene was used as the housekeeping gene in the analysis.

For this experiment, groups of 10 male BALB/c mice were infected by tail vein injection with the following *Paracoccidioides* isolates: Pb18 (*P. brasiliensis*) and Pb01 (*P. lutzii)*. The blood of the animals was collected for RNA extraction (Bailão et al., 2006) after 10 min and 1 h of infection, and gene expression was investigated. In this study, we analyzed the expression of adhesins at earlier time points (10 min and 1 h) than those evaluated for adhesion studies (2 and 5 h) to determine the expression of adhesins in response to the first contact of the fungus with the host (infected BALB/C mice) because adhesins are molecules of great importance during the initial contact of the fungus with the host.

RNA from the fungi recovered from mouse blood was extracted using the TRIzol method (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA synthesis was performed using reverse transcriptase (RevertAid™ H Minus Reverse Transcriptase, Fermentas, Canada) and 1 μg of total RNA.

The reaction mixtures contained 1 μL of cDNA, 12.5 μL of Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, Canada), and 0.5 μM of each primer, and the volume was brought to 25 μL with nuclease-free water. The reaction program was 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by annealing and synthesis at 60°C for 1 min. Following the PCR, a melting curve analysis was performed, which confirmed that the signal corresponded to a single PCR product. Reactions were performed in an Applied Biosystems 7500 cycler. The data were analyzed using the $2^{-\Delta\Delta CT}$ method. The cycle threshold values for the triplicate PCRs of each RNA sample were averaged, and then the $2^{-\Delta\Delta CT}$ values were calculated using the control gene 60S ribosomal L34, which is a constitutive housekeeping gene that is widely used as a control in different studies of paracoccidioidomycosis (Felipe et al., 2005a; Bailão et al., 2006, 2007; Brito et al., 2011; Parente et al., 2011;

TABLE 1 | Primers used for the real-time PCR assays.

Gene	Primers	Product length (bp)
Enolase	Sense 5′-TAGGCACCCTCACTGAATCC-3′ Antisense 5′-GCTCTCAATCCCACAACGAT-3′	130
GADPH	Sense 5′-AAATGCTGTTGAGCACGATG-3′ Antisense 5′-CTGTGCTGGATATCGCCTTT-3′	129
GP43	Sense 5′-CTTGTCTGGGCCAAAAACTC-3′ Antisense 5′-CGGGACTGGGAGTGAGATAT-3′	135
Malate synthase	Sense 5′-GTTCCCTTCATGGATGCCTA-3′ Antisense 5′-TCTTTGATGGGGATTTGAGC-3′	101
Triosephosphate isomerase	Sense 5′-CCTTACGGCAGAATGACGTT-3′ Antisense 5′-GCCATTTCCATGTCAGGTCT-3′	118
14-3-3	Sense 5′ -GTTCGCTCTTGGAGACAAGC-3′ Antisense 5′ -AGCAACCTCAGTTGCGTTCT-3′	76
L34 (endogenous gene)	Sense 5′-TGTCTACACTGCGCAAGGAC-3′ Antisense 5′-ATGTGTTGGTGGGAGAGGAG-3′	149

Peres da Silva et al., 2011; Lima et al., 2014). A sample that contained all reagents except *P. brasiliensis* cDNA was used as a negative control. After 40 rounds of amplification, no PCR products were detected in this reaction. Two biological samples were obtained for each studied situation and for each sample, and three independent experiments were performed. As a control experiment, an RNA sample was obtained from the blood of mice not infected with *P. brasiliensis*. Before beginning the experiments, all of the primers were evaluated to assess the efficiency of the amplifications by analyzing the standard curves using serial dilutions of cDNA as a template and determining the slope value. Subsequently, we used this value to evaluate the efficiency using the formula Efficiency $= 10^{(-1/\text{slope})}$-1. After the experiments, the amplification products (in 1.5% agarose gels) and the dissociation curves were analyzed to ensure the amplification of only one PCR product. Statistical analyses were performed using One-Way ANOVA with Tukey's coefficient. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).

Analysis of the Relationship between Adhesion and Virulence in Different Species of the Genus *Paracoccidioides*

For this analysis, aliquots (5×10^6 cells/ml) of each isolate of the genus were intratracheally inoculated into C57BL/6 male mice. The mice were anesthetized by administration of a combination of 250 μL of 10 mg/kg xylazine and 80 mg/kg ketamine hydrochloride. A small incision was made in the trachea region, and 0.05 mL of fungal suspension inoculum was injected using a 1.0 mL syringe. The incision was sutured, and the animals were kept warm to avoid hypothermia due to the action of the anesthetic. After inoculation, the mice were observed for mortality until 200 days post-infection. Each group contained eight mice, and a control group was inoculated with only PBS. For the survival curves, the statistical analyses were performed using the Mantel-Cox test in GraphPad Prism software, and the p-value was set at $p < 0.05$.

Evaluation of the Role of the Two Major Expressed Adhesins, 14-3-3 and Enolase, in the Adhesion of the *Paracoccidioides* Genus to A549 Pneumocytes

This experiment was made in the same way as described in the section Adhesion to A549 pneumocytes of *P. brasiliensis* and *P. lutzii* with a modification. After the treatment of the *Paracoccidioides* spp. cells with 10 uM CFSE, the cells were washed, resuspended in PBS and treated with 14-3-3 and enolase rabbit antisera (1:100) for 1 h at 37°C. After this new treatment, the cells were washed, resuspended in PBS and added to A549 pneumocytes culture for 2 and 5 h. These experiments were also performed in triplicate, with three independent experiments for each species. Statistical analyses were performed using One-Way ANOVA with Tukey's coefficient. As controls, we used *Paracoccidioides* spp. without any treatment and treated with rabbit antisera (1:100) for 1 h at 37°C. The results of the statistical analyses were considered significant when the p-value was <0.05. Analyses and the construction of graphs were performed using Prism 5 software (GraphPad Software Inc.).

Evaluation of the Role of the Two Major Expressed Adhesins, 14-3-3 and Enolase, in the Virulence of the *Paracoccidioides* Genus Using *G. mellonella*

For this analysis, *P. brasiliensis* (Pb18) and *P. lutzii* (Pb01) were treated with 14-3-3 and enolase rabbit antisera (1:100) for 1 h at 37°C. The 14-3-3 and enolase rabbit antisera used in this study were obtained and used in three previous studies performed by our group (Donofrio et al., 2009; Marcos et al., 2012; da Silva et al., 2013). After treatment, the inocula were washed 3 times with PBS and inoculated into the larvae of *G. mellonella*. The rearing of *G. mellonella* was performed according to Ramarao et al. (2012). The larvae were fed wax and pollen and maintained at 25°C until they reached 100–200 mg in weight. Larvae without color alterations and with the appropriate weight were selected in groups of 16 larvae, placed in Petri dishes and incubated at 37°C in the dark on the night before the experiments. The larvae

were inoculated through the last left pro-leg using a 10 μL Hamilton syringe (Hamilton, USA) after cleaning the pro-leg with 70% ethanol.

A total of 5×10^6 cells were injected into each larva. Larval death was recorded over the next 7 days and was based on lack of movement after manipulation with forceps. For this experiment, 6 different controls were used: (1) PBS, (2) larvae treated with 14-3-3 rabbit antisera alone (not infected with fungi), (3) larvae treated with enolase rabbit antisera alone (not infected with fungi), (4) larvae treated with rabbit antisera alone (no infection), (5) larvae infected with *P. brasiliensis* and treated with rabbit antisera for 1 h at 37°C, (6) larvae infected with *P. lutzii* and treated with rabbit antisera for 1 h at 37°C, (7) larvae treated with *P. brasiliensis* alone and (8) larvae treated with *P. lutzii*.

For the survival curves, three independent experiments were performed. The statistical analyses were performed using the Mantel-Cox test in GraphPad Prism software, and the *p*-value was set at $p < 0.05$.

The influence of antisera on the survival and morphology of the fungi was evaluated to analyze how these factors interfere with the results found in the adhesion inhibition assays and survival curves. For this, *Paracoccidioides* spp. Pb18 and Pb01 were grown for 7 days in BHI with 2% glucose and with or without 14-3-3, enolase or rabbit antisera at a titer of 1:100. Every 24 h, aliquots of the fungi were obtained, and the morphology and viability were analyzed. To analyze morphology, the fungi were fixed with 1% paraformaldehyde, stained with calcofluor white stain (40 μL/mL) (Sigma-Aldrich) and observed using fluorescence microscopy. The viability of the cells was assessed by measuring metabolic activity using a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide (XTT) (Sigma) reduction assay as described by Meshulam et al. (1995).

Results

Adhesion to A549 Cells of *Paracoccidioides* spp.

The results of the adhesion assay with the different species are presented in **Figure 1**. The adhesion rates were significantly different ($p < 0.05$) when comparing *P. brasiliensis* and *P.* lutzii. After 2 h of interaction, the percentage of adhesion of *P. brasiliensis* was approximately 42.3%, while the adhesion of the *P. lutzii* isolate was approximately 36.8%. After 5 h of infection, the difference in the adhesion rates was still significantly different, with rates of approximately 45.8% in the *P. brasiliensis* isolate and approximately 39.7% in the *P. lutzii* isolate. The adhesion rate may reflect the virulence of each isolate because adhesion is an essential step for successful infection by fungi of the *Paracoccidioides* genus.

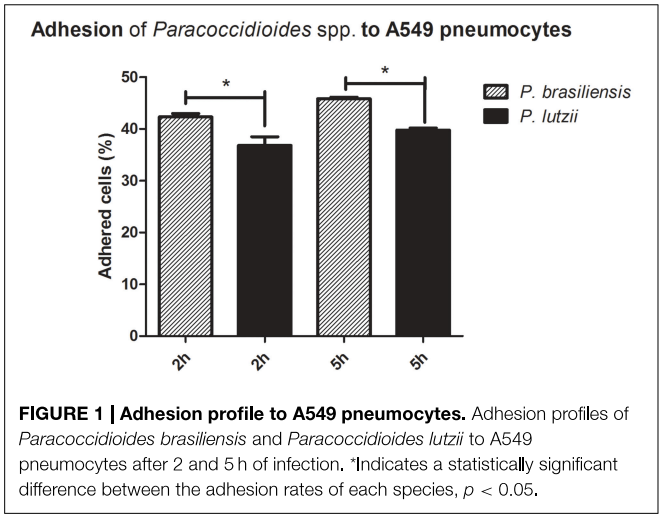


FIGURE 1 | Adhesion profile to A549 pneumocytes. Adhesion profiles of *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* to A549 pneumocytes after 2 and 5 h of infection. *Indicates a statistically significant difference between the adhesion rates of each species, $p < 0.05$.

Adhesion of *Paracoccidioides* spp. to the ECM Components Laminin, Fibronectin, and Type I and Type IV Collagen

These assays were performed with Pb18 (*P. brasiliensis*) and Pb01 (*P. lutzii*) isolates that were placed into contact with ECM components (laminin, fibronectin, and collagens type I and type IV), after which adherent cells were removed and counted by flow cytometry. Three independent experiments were performed in triplicate and statistically evaluated (**Figure 2**).

The two species appeared to have different affinities for certain extracellular matrix components. Pb18 (*P. brasiliensis*) showed increased adhesion to fibronectin, while Pb01 (*P. lutzii*) was more adherent to type I and IV collagen.

Expression Analysis of Genes Encoding Adhesins by Real-Time PCR

Before we analyzed the relative expression, the efficiency of the amplifications was evaluated. For this analysis, we had to determine the value of the slope for each tested primer, and we found that the values were close to −3.32 (L34: −3.4; GP43: −3.24; ENO: −3.26; TPI: −3.24; GAPDH: −3.22; 14-3-3: −3.24; MLT: −3.25); thus, the efficiency was greater than 95% (L34: 96%; GP43: 100%; ENO: 100%; TPI: 100%; GAPDH: 100%; 14-3-3: 100%; MLT: 100%). In addition, after the amplification, the amplicons were checked by running them on agarose gels to confirm the size of the expected products. The melting curves presented only one peak, indicating that only one product was amplified (**Supplementary Figure 1**).

The relative expression levels of different adhesins in the different *Paracoccidioides* species are presented in **Figure 3**. Adhesin expression varies, with some adhesins appearing to be more important than others for infection by the different species. Generally, *P. brasiliensis* adhesins are more highly expressed than *P. lutzii* adhesins. Specifically, enolase and 14-3-3 seem to be the most important adhesins for the *Paracoccidioides* genus during its interaction with the host.

During the initial step of the interaction (10 min), only the TPI adhesin was down-regulated in *P. brasiliensis*, while all of the others analyzed were up-regulated during this step. After 1 h of this interaction, the level of expression was still significantly increased in *P. brasiliensis,* showing the importance of these molecules during the interaction of this species with the host. For *P. lutzii*, we observed the opposite, with most of the adhesins down-regulated during the initial step (10 min) of interaction with the host. However, we note that the TPI adhesin was up-regulated and therefore

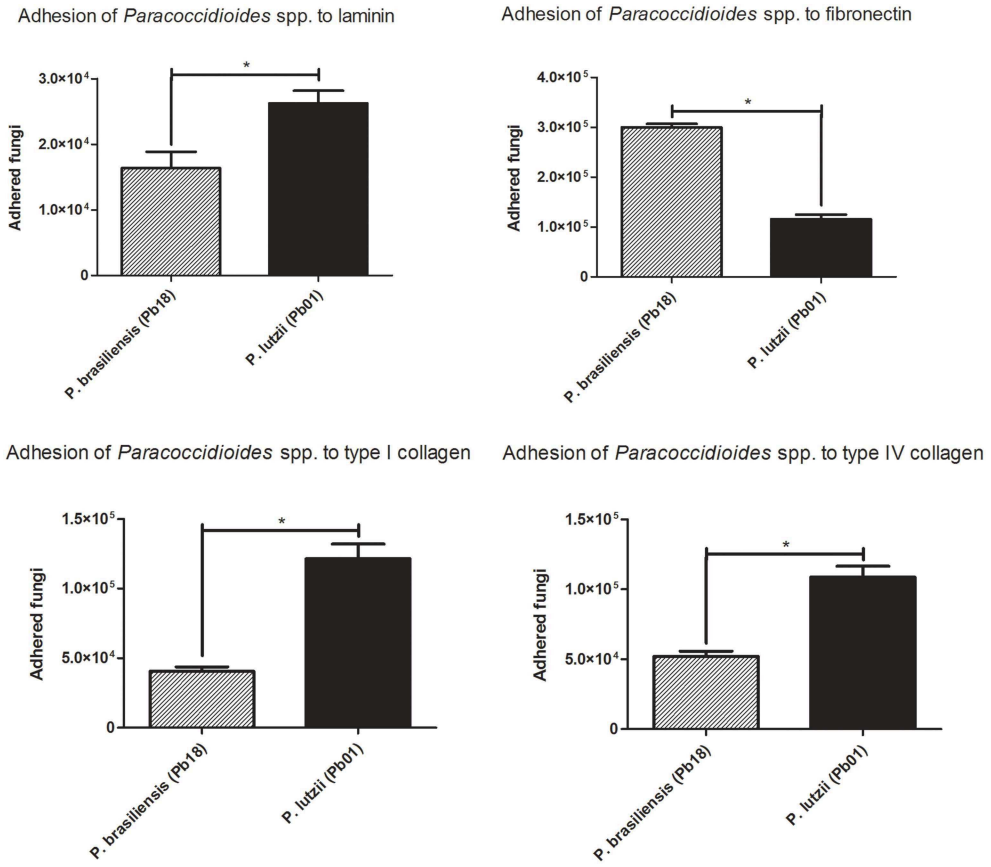


FIGURE 2 | Adhesion profile to ECM components. Adhesion profiles of the different species of the genus *Paracoccidioides* to the ECM components (laminin, fibronectin, Type I collagen and Type IV collagen) after 2 h of infection. *Indicates a statistically significant difference in the adherence rate, $p < 0.05$.

seems to be one of the most important adhesins during the initial process of infection. After 1 h, we were able to observe the same pattern observed in *P. brasiliensis*, namely, a significantly increase in the expression of adhesins.

Survival Curve of C57BL/6 Mice for the Evaluation of the Adhesion/Virulence Relationship in the Genus *Paracoccidioides*

The C57BL/6 mouse strain was chosen because this lineage is more sensitive to *Paracoccidioides* infection and is frequently used for studies examining paracoccidioidomycosis (de Pádua Queiroz et al., 2010; Bernardino et al., 2013; Costa et al., 2013; da Silva et al., 2013).

After 200 days of infection, we observed that 4 individuals infected with *P. brasiliensis* died, while only 1 individual infected with *P. lutzii* died, resulting in mouse survival rates of 50 and 90%, respectively (**Figure 4**).

Evaluation of the Role of the Two Major Expressed Adhesins, 14-3-3 and Enolase, in the Adhesion of the *Paracoccidioides* Genus to A549 Pneumocytes

It is important to know how these adhesins in fact influence in the adhesion. For this we proposed an experiment where we blocked the two major expressed adhesins 14-3-3 and enolase and, with an adhesion assay, we could measure how much these adhesins influences in the infection process of *Paracoccidioides* spp. After blocking 14-3-3 and enolase we could observe, in 2 and 5 h, that the capacity to adhere to pneumocytes was affected in *Paracoccidioides* spp. (**Figure 5**). After 2 and 5 h, we could observe that the blocking of 14-3-3 adhesin, lead to a decrease of 19.3 and 24.8 % of *P. brasiliensis* adhesion, respectively. In the case of enolase, adhesion was reduced 8.9 and 10.4% after 2 and 5 h, respectively. For *P. lutzii* we could observe that blocking of 14-3-3 led to 9.8 and 6.2% of the reduction of adhesion after 2 and 5 h. In the case of enolase, adhesion was reduced 9.9 and 5.9% after 2 and 5 h, respectively. These results show that both adhesins are important for full virulence in *Paracoccidioides* spp.

Evaluation of the Role of the Two Major Expressed Adhesions, 14-3-3 and Enolase, in the Virulence of the Genus *Paracoccidioides* Using *G. mellonella*

If adhesin expression actually interferes with the virulence of the fungus, blockage of adhesin should affect virulence. To evaluate this hypothesis, we decided to block two of the most highly expressed adhesins in *Paracoccidioides* spp. (based on real-time PCR experiments), 14-3-3 and enolase, using polyclonal

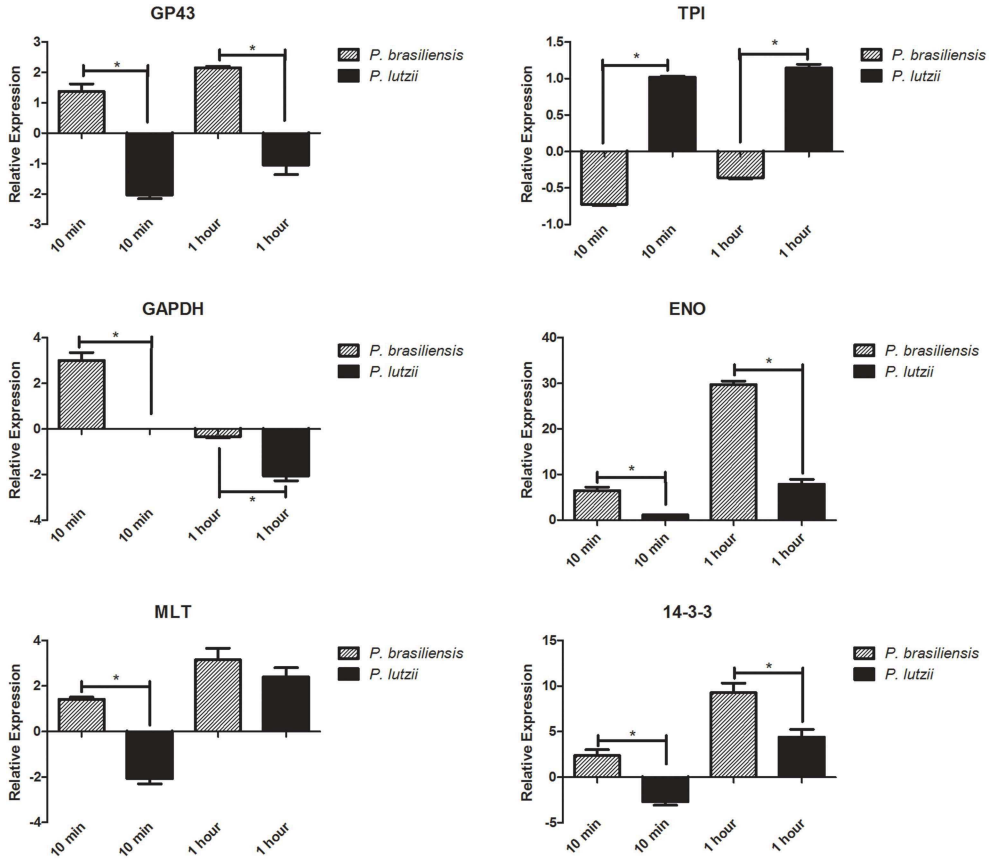


FIGURE 3 | Relative expression of adhesins. Relative expression of different genes encoding adhesins in the genus *Paracoccidioides*. *Indicates a statistically significant difference in expression level, $p < 0.05$. The graphs show the normalized value for adhesin gene expression relative to a suitable reference (fungi without contact with the host), which was assigned an arbitrary value of 1.

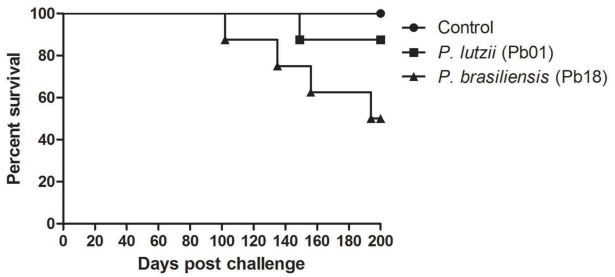


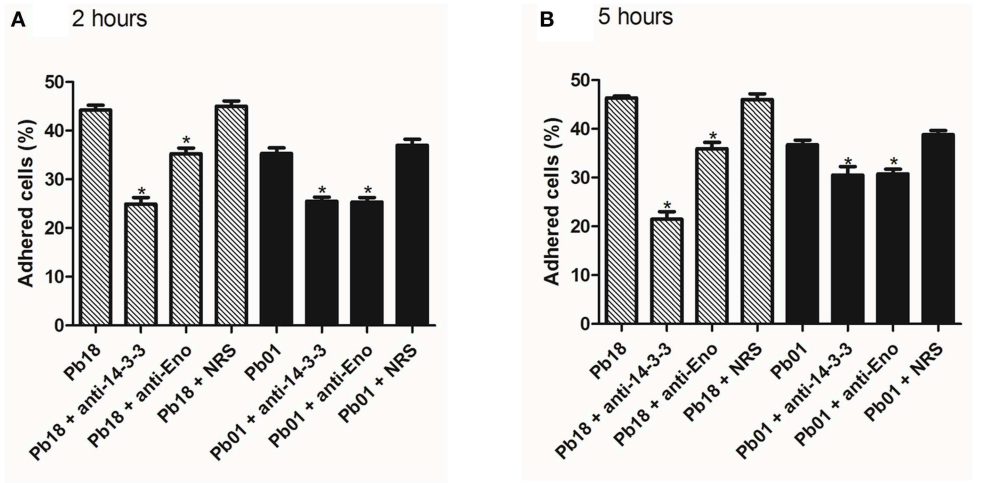
FIGURE 4 | Virulence of the genus *Paracoccidioides*. Survival curves of C57BL/6 mice infected with *P. brasiliensis* and *P. lutzii* observed for 200 days after infection, showing an increased ability of *P. brasiliensis* to kill mice, with a statistically significant difference relative to the control ($p < 0.05$).

antibodies. For this experiment, *P. brasiliensis* and *P. lutzii* were separately treated with 14-3-3 and enolase rabbit antisera (1:100) for 1 h at 37°C. Then, these fungi (5×10^6 cells/larva) were used to infect *G. mellonella* larvae. The virulence of these treated fungi was evaluated for 7 days after fungal challenge. This fungal concentration was previously determined by measuring survival curves with different inoculum sizes for both *P. brasiliensis* and *P. lutzii* (data not shown). Treatment of *P. brasiliensis* and *P. lutzii* with 14-3-3 and enolase polyclonal antibodies led to significant reductions in virulence, as measured by mortality ($p < 0.05$). We observed survival rates of 68.7 and 31.2% for *P. brasiliensis* and 20 and 22% for *P. lutzii* after treatment with 14-3-3 and enolase polyclonal antibodies, respectively (**Figure 6**). Control larvae showed 100% survival at the end of the 7-day experiment.

These results reflect the influence of these adhesins on the virulence of *Paracoccidioides* spp. because our experimental controls showed us that treating the fungi with the different antisera did not influence survival, with greater than 90% of cells remaining viable for all 7 days of the experiment. Additionally, the morphology of the fungi was not altered during the experiment. All of the results of these control analyses are presented in **Supplementary Figure 2**. **Table 2** shows the standard deviations for each mean value in the graphs.

Discussion

Studies have demonstrated the ability of *Paracoccidioides* spp. to adhere and invade (Mendes-Giannini et al., 1994), and these characteristics vary depending on the isolate (Hanna et al., 2000).



2 hours adhesion assay statistics summary	
Tukey's Multiple Comparison Test	**Significant? P < 0.05?**
Pb18 vs Pb18 + anti-14-3-3	Yes
Pb18 vs Pb18 + anti-Eno	Yes
Pb18 vs Pb18 + NRS	No
Pb18 vs Pb01	Yes
Pb18 + anti-14-3-3 vs Pb18 + anti-Eno	Yes
Pb18 + anti-14-3-3 vs Pb18 + NRS	Yes
Pb18 + anti-14-3-3 vs Pb01 + anti-14-3-3	No
Pb18 + anti-Eno vs Pb18 + NRS	Yes
Pb18 + anti-Eno vs Pb01 + anti-Eno	Yes
Pb01 vs Pb01 + anti-14-3-3	Yes
Pb01 vs Pb01 + anti-Eno	Yes
Pb01 vs Pb01 + NRS	No
Pb01 + anti-14-3-3 vs Pb01 + anti-Eno	No
Pb01 + anti-14-3-3 vs Pb01 + NRS	Yes
Pb01 + anti-Eno vs Pb01 + NRS	Yes

5 hours adhesion statistics summary	
Tukey's Multiple Comparison Test	**Significant? P < 0.05?**
Pb18 vs Pb18 + anti-14-3-3	Yes
Pb18 vs Pb18 + anti-Eno	Yes
Pb18 vs Pb18 + NRS	No
Pb18 vs Pb01	Yes
Pb18 + anti-14-3-3 vs Pb18 + anti-Eno	Yes
Pb18 + anti-14-3-3 vs Pb18 + NRS	Yes
Pb18 + anti-14-3-3 vs Pb01 + anti-14-3-3	Yes
Pb18 + anti-Eno vs Pb18 + NRS	Yes
Pb18 + anti-Eno vs Pb01 + anti-Eno	No
Pb01 vs Pb01 + anti-14-3-3	Yes
Pb01 vs Pb01 + anti-Eno	Yes
Pb01 vs Pb01 + NRS	No
Pb01 + anti-14-3-3 vs Pb01 + anti-Eno	No
Pb01 + anti-14-3-3 vs Pb01 + NRS	Yes
Pb01 + anti-Eno vs Pb01 + NRS	Yes

FIGURE 5 | Influence of adhesins in the adhesion profile of *Paracoccidioides* spp. to pneumocytes A549. Adhesion profiles of *P. brasiliensis* and *P. lutzii* treated with 14-3-3 and enolase antibodies to A549 pneumocytes after **(A)** 2 h and **(B)** 5 h of infection. As controls, we used *P. brasiliensis* and *P. lutzii* treated with rabbit serum (NRS), and without any treatment. *Indicates statistically significant differences between the adhesion rates, $p < 0.05$. The tables below each graph are summaries of adhesion assay statistics.

Previous studies have demonstrated that Pb18, now classified as belonging to the S1 phylogenetic species, is the most pathogenic strain in animals (Singer-Vermes et al., 1989) and is more adherent to Vero cells (Hanna et al., 2000). Additionally, after several subcultures, Pb18 loses its adhesion ability, indicating the important relationship between virulence and adhesion. However, upon re-isolation from animals or cell culture, this fungus recovers its ability to adhere to and invade epithelial cells (Andreotti et al., 2005).

In our study, we observed that adhesion was significantly higher for the Pb18 isolate of *P. brasiliensis* than for the Pb01 isolate of *P. lutzii*. The adhesion process reflects the virulence of the isolates because efficient adhesion will culminate in rapid invasion of the host cells, allowing the fungus to escape the host immune system, establish infection, and, in the case of *Paracoccidioides* spp., cause systemic mycosis. After 5 h of infection, the adhesion rate continues to increase, indicating a high capacity to adhere to host cells, as previously observed by our group (Hanna et al., 2000).

These differences in adhesion in different species may reflect differences in the molecules used during interactions with the host. Molecules with greater affinity for different types of ECM components may be associated with the virulence of each isolate and the establishment of the disease in different tissues. Different cell types produce components of the matrix in different quantities, which facilitates the interaction of the fungus with the host depending on the site of the infection. The ability of a microorganism to adhere and invade is recognized as an important factor in pathogenicity. Adhesion implies that the fungus recognizes ligands on the surface of the host cell or a component of the ECM. The adhesion mechanism has been extensively studied in bacteria and pathogenic fungi (de Groot et al., 2013), such as *Candida albicans* (Nobile et al., 2008; Murciano et al., 2012; Puri et al., 2014; Silva et al., 2014; Formosa et al., 2015), *Aspergillus fumigatus* (Upadhyay et al., 2009; Al Abdallah et al., 2012; Xu et al., 2012; Zhao et al., 2012), *Sporothrix schenckii* (Ruiz-Baca et al., 2009; Teixeira et al., 2009b; Sandoval-Bernal et al., 2011), *Coccidioides immitis* (Hung et al., 2002), *Histoplasma capsulatum* (Taylor et al., 2004; Suárez-Alvarez et al., 2010; Pitangui et al., 2012) and *Penicillium marneffei* (Srinoulprasert et al., 2006, 2009; Lau et al., 2013). In a previous study, our group demonstrated that *P. brasiliensis* interacts with human

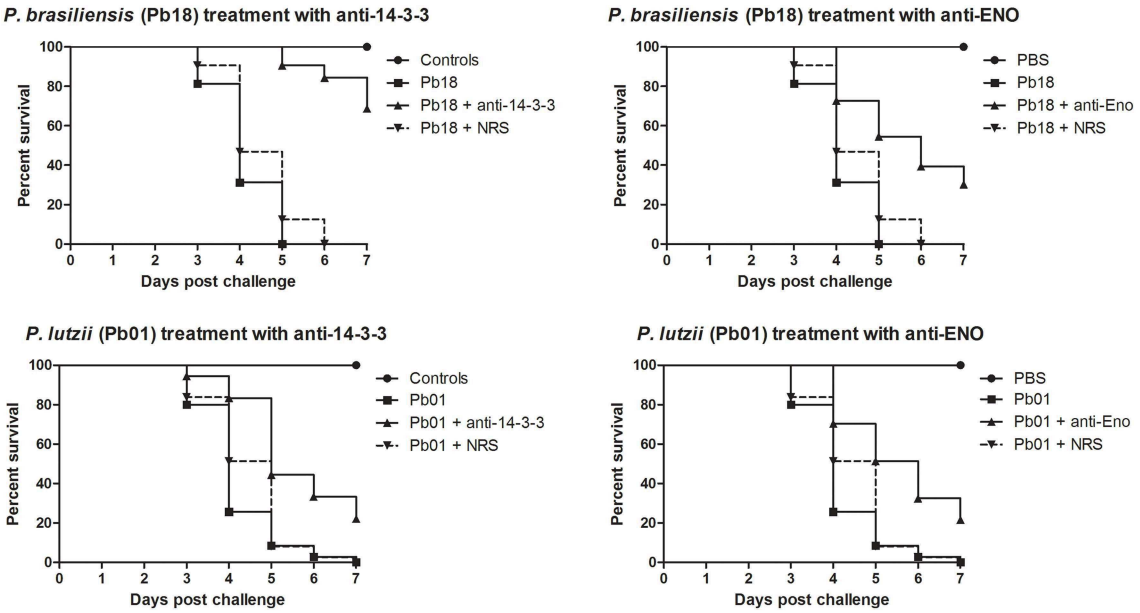


FIGURE 6 | Influence of adhesins on the virulence of the genus *Paracoccidioides*. Survival curve of *G. mellonella* larvae challenged with *P. brasiliensis* and *P. lutzii* and treated with 14-3-3 and enolase antibodies. Survival was evaluated for 7 days after challenge and showed a reduction in the virulence of the fungi when treated with the antibodies, with a statistically significant difference compared to untreated *P. brasiliensis* and *P. lutzii* ($p < 0.05$). There were 6 different controls: (1) PBS, (2) larvae treated with 14-3-3 antibody alone (not infected with fungi), (3) larvae treated with enolase antibody alone (not infected with fungi), (4) larvae treated with rabbit serum alone (no infection), (5) larvae infected with *P. brasiliensis* and treated with rabbit serum for 1 h at 37°C, and (6) larvae infected with *P. lutzii* and treated with rabbit serum for 1 h at 37°C. All of the controls showed 100% larval survival after the 7-day experiments.

fibronectin (Mendes-Giannini et al., 2006), which confirmed the results of the present study, although there was apparently greater adhesion to laminin. However, these previous assays were performed with extracts, whereas we used whole fungal cells in the present study.

Adhesion is related to the expression of adhesins, and several adhesins have been described in *Paracoccidioides* spp. (Mendes-Giannini et al., 2000, 2008) however, these molecules have not been previously studied to determine their role in the dynamics of infection. In the present study, we analyzed the expression of adhesins at earlier times (10 min and 1 h) than those used for adhesion studies (2 and 5 h) because we aimed to determine whether the fungi respond to host contact and when adhesin expression occurs before the fungi begin the invasion process.

The expression of adhesins in the different species was not constant, and a wide range of expression was observed among the isolates, including a lack of expression. We observed greater expression of certain adhesins, depending on the species and the duration of fungal contact.

The increased expression of GP43 was observed only for *P. brasiliensis* (approximately 1.5-fold higher expression at 10 min and 2.3-fold higher expression at 1 h), while its expression was reduced in *P. lutzii* (approximately 2-fold less expression at 10 min and 1-fold at 1 h). These data are extremely important for the study of paracoccidioidomycosis because the GP43 protein, aside from its role as an adhesin, is the main serological marker for this disease (Puccia and Travassos, 1991). All phylogenetic species cause paracoccidioidomycosis, but a lack of expression of this protein may lead to inaccuracy in serological diagnosis, as demonstrated in some studies (Rocha et al., 2009; Batista et al., 2010; Machado, 2011; Puccia et al., 2011). Our study confirms the data that have already demonstrated the differential role of GP43 in *P. lutzii* specifically that it is not involved in the adhesion of the fungus in this species, and shows that new serological markers for the disease should be screened to avoid making diagnostic mistakes when patients are infected with *P. lutzii* isolates. Additionally, these data confirm that the expression of GP43 is of extreme importance for *P. brasiliensis* virulence, reflecting the importance of this molecule for the diagnosis of patients affected with paracoccidioidomycosis caused by this species.

Another important finding in our study is related to the increase in enolase adhesin expression for the two species, which was approximately 8-fold at 10 min and approximately 35-fold after 1 h of infection with *P. brasiliensis* and was approximately 1-fold after 10 min and approximately 10-fold after 1 h of infection with *P. lutzii*. Recent studies have shown the importance of this protein in *Paracoccidioides* adhesion and virulence. Donofrio et al. (2009) first demonstrated that enolase is a fibronectin adhesin for *P. brasiliensis*, and Nogueira et al. (2010) subsequently described its binding to laminin and collagen type I in addition to fibronectin. Moreover, enolase expressed on the surface of *P. lutzii* acts as a plasminogen receptor, and through this receptor, the fungus can acquire proteolytic activity because of the plasmin generated by this binding event. Plasmin is

TABLE 2 | Standard deviations of the *Galleria mellonella* survival curves.

(A) Days	Controls		Pb 18		Pb 18 + Anti-14-3-13		Pb 18 +Anti-ENO		Pb 18 + NRS	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	100		100		100		100		100	
3	100		81.25	6.90	100		100		90.63	5.15
4	100		31.25	8.19	100		72.73	7.75	46.88	8.82
5	100		0	0	90.6	5.2	54.55	8.67	12.50	5.85
6	100		0	0	84.4	6.4	39.39	8.51	0	0
7	100		0	0	68.8	8.2	30.30	8.00	0	0

(B) Days	Controls		Pb01		Pb01 + Anti-14-3-3		Pb01 + Anti-ENO		Pb01 + NRS	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	100		100		100		100		100	
3	100		80.00	6.76	94.44	3.82	100		83.78	6.06
4	100		25.71	7.39	83.33	6.21	70.27	7.51	51.35	8.22
5	100		8.57	4.73	44.44	8.28	51.35	8.22	8.11	4.49
6	100		2.86	2.82	33.33	7.86	32.43	7.70	2.70	2.67
7	100		0.00	0.00	22.22	6.93	21.62	6.77	0.00	0.00

a key enzyme of the plasminogen system and contributes to the degradation of matrix constituents. This contributes to the pathogenicity of *P. lutzii* because it facilitates tissue invasion by the pathogen. Marcos et al. (2012) demonstrated that fibronectin was the major enolase binder, and it was found at high levels in the fungal cell wall, thereby contributing to *P. brasiliensis* adhesion to the host.

A high level of expression of 14-3-3 adhesin was observed in *P. brasiliensis* after 10 min of interaction, and a significant increase in the expression of 14-3-3 was observed after 1 h for the two species (approximately 12-fold for *P. brasiliensis* and 6-fold for *P. lutzii*). This protein has recently been identified as an important factor in the interaction between *Paracoccidioides* and its host. In a recent study, da Silva et al. (2013) demonstrated that a significant increase in this protein occurs on the pathogen cell wall during its interaction with A549 epithelial cells. This increase suggests an important role for this protein in the fungus-host interaction, leading to a cellular immune response that is important for the success of the fungus in the microenvironment of the host cells. In addition, Vallejo et al. (2011, 2012b) verified that this protein is transported by *P. brasiliensis* vesicles and is secreted into the culture supernatant during *ex vivo* interactions, as described by da Silva et al. (2013).

We observed an increase of triosephosphate isomerase expression in *P. lutzii* of approximately 1-fold after 10 min and 1 h. This protein has only been characterized as an adhesin in isolate Pb01 from *P. lutzii* (Pereira et al., 2007), and it has not been studied in other isolates of the genus *Paracoccidioides*.

Glyceraldehyde 3-phosphate dehydrogenase has also been characterized as an adhesin of *P. lutzii* (Barbosa et al., 2006). In our study, we observed the expression of this adhesin during early interactions between *P. brasiliensis* and the host (an increase of approximately 3.5-fold). For both species, the expression decreased after an hour of interaction (approximately 0.5-fold less expression for *P. brasiliensis* and 2.5-fold less expression for *P. lutzii*), revealing the importance of this protein in early infections with *P. brasiliensis*. Although this finding is controversial, the conditions of our experiment may have influenced these results because this adhesin was described in an *in vitro* experiment, while our study was carried out *in vivo*.

Malate synthase has been described as an adhesin for Pb01 (*P. lutzii*) (da Silva Neto et al., 2009), binding to fibronectin and type I and type IV collagen. Moreover, (de Oliveira et al, 2013) demonstrated that *Paracoccidioides* malate synthase interacts with proteins of different molecular categories, such as those involved in cellular transport, protein biosynthesis, protein degradation and modification and signal transduction, suggesting that this protein plays pleiotropic roles in fungal cells. In the present study, it was expressed less during the early interaction of *P. lutzii* (approximately 2-fold less expression) and of *P. brasiliensis* (expressed approximately 1.5-fold more), but its expression was increased in both species after 1 h of interaction (approximately 4-fold for *P. brasiliensis* and 3-fold for *P. lutzii*).

Apparently, the enolase and 14-3-3 were the most highly expressed adhesins. We observed that the expression of adhesins depends on the duration of the interaction between the host and the fungus. The highest levels of expression for most adhesins were observed in *P. brasiliensis*. This study presents a new perspective on the complex interaction between the *Paracoccidioides* genus and the host and demonstrates the importance of studying different species during the course of infection to understand

the molecular arsenal used by the fungus to ensure pathogenic success.

A survival curve was constructed, and *P. brasiliensis* was shown to be more virulent than *P. lutzii*. *P. brasiliensis* was able to kill 50% of the group, while *P. lutzii* killed only 10%. The *Paracoccidioides*-host interaction may cause the induction of apoptosis in the host cells (Mendes-Giannini et al., 2004; Del Vecchio et al., 2009), and this induction can facilitate the establishment and dissemination of the infection in the host. In this way, because *P. brasiliensis* adhere in a more efficient way to pneumocytes than *P. lutzii*, this interaction in an *in vivo* model could lead to the efficient establishment of infection by *P. brasiliensis*, confirming the survival curve observed. These results show that there is an apparent relationship between adherence and virulence in *Paracoccidioides*.

The adhesion of *Paracoccidioides* spp. after blocking 14-3-3 and enolase is significantly affected showing us the importance of these molecules to the infection process of *Paracoccidioides* spp. The 14-3-3 seems to have more influence in this process for *P. brasiliensis* than *P. lutzii*, while enolase, besides seems to have less influence in the adhesion process, the differences found in the adhesions rates are statistically significant. In previously studies, da Silva et al. (2013) made an adhesion inhibition assay of *P. brasiliensis* (Pb18) to pneumocytes A549 and could observe an inhibition in the infection after the treatment of the fungi with the same 14-3-3 antisera, at 2 and 24 h. In a similar way, Donofrio et al. (2009) treated Pb18 cells with enolase antisera and could observe that the adhesion was markedly inhibited by pneumocytes A549 cells after 2 h and 5 h of interaction.

When virulence was evaluated after blocking 14-3-3 and enolase adhesins in *G. mellonella*, we could clearly see that this blocking led to a reduction in the virulence of *P. lutzii* and *P. brasiliensis,* confirming the relationship between adhesins and virulence. After blocking with anti-adhesin antibodies, *P. brasiliensis* virulence was more affected than *P. lutzii* virulence. These data corroborate the real-time PCR results, showing that *P. brasiliensis* expressed higher levels of adhesins than *P. lutzii,* and this elevated expression could be responsible for the higher virulence of *P. brasiliensis* when compared with *P. lutzii*. Besides this, these results also corroborates with the adhesion inhibition assays after blocking 14-3-3 and enolase where we could observe a higher influence of the 14-3-3 in the adhesion of *P. brasiliensis*, however, even with less influence, these two adhesins impact in the virulence of *P. brasiliensis* and *P. lutzii*.

With adhesion blocked, larvae were able to resist the fungal infection and survive. The immune system of *G. mellonella* presents high homology with mammalian organisms and for this reason is considered a suitable model for studying the fungal immune response (Fuchs and Mylonakis, 2006). The cuticle is the first barrier to microorganisms (Champion et al., 2009); moreover, this insect has humoral and cellular defenses. The humoral response is carried out by phenoloxidase, reactive oxygen species and antimicrobial peptides present in the hemolymph, in addition to six different types of cells with phagocytic capacity (Vilmos and Kurucz, 1998; Bergin et al., 2005). Other cellular reactions of the immune system realized by the hemocytes are nodulation and encapsulation; these types of cellular responses occur when the phagocytic cells are unable to phagocytize due to the size of the microorganism, for example (Jiravanichpaisal et al., 2006).

In our results, adherence to A549 pneumocytes by *P. brasiliensis* was more pronounced than in *P. lutzii*. When we examined adhesion to ECM components, we observed that *P. lutzii* was able to adhere more efficiently to three components of the extracellular matrix: laminin, type I collagen and type IV collagen. However, *P. brasiliensis* adhered more efficiently to fibronectin. Fibronectin is an ECM component that is highly important in lung tissue, which is the preferred site of *Paracoccidioides* interaction with the host, because fungal infection occurs through the inhalation of conidia. This makes the lungs the first site of contact between the fungus and the host. Furthermore, *P. brasiliensis* exhibited higher levels of adhesin expression when compared to *P. lutzii,* resulting in greater adherence of these species. These data, when analyzed in relation to the survival curves, make it clear that the greater ability of *P. brasiliensis* to express adhesins leads to greater adhesion and results in increased virulence *in vivo*. Thus, there is a clear relationship between adhesion ability, adhesin expression and fungal virulence in the genus *Paracoccidioides*.

Acknowledgments

This work was supported by the Brazilian organizations Fundação de Apoio à Pesquisa do Estado de São Paulo (FAPESP) 2011/18038-9 and Rede Nacional de Métodos Alternativos - Conselho Nacional de Desenvolvimento Científico e Tecnológico (RENAMA-CNPq) 403586/2012-7. We thank Daniella Sayuri Yamazaki for support with the animal experiment and Dr. Isabel Cristiane da Silva for all the support with A549 pneumocytes culture.

Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00303/abstract

Supplementary Figure S 1 | Specific amplification of the studied adhesin genes by real-time PCR. (A) Melting curve analysis of all of the primers used showing single peaks. **(B)** Agarose gel electrophoresis of the PCR products showing single bands.

Supplementary Figure S 2 | Influence of antisera treatment on the survival and morphology of the fungi. Analysis of the influence of fungi treatment with 14-3-3, enolase and NRS antisera. **(A)** and **(C)** Viability of *P. lutzii* and *P. brasiliensis*, respectively, after treatment with 14-3-3, enolase and NRS antisera. No significant differences were found between the different treatments and the untreated controls. (B) and (D) Analysis of the morphology of *P. lutzii* and *P. brasiliensis*, respectively, after treatment with 14-3-3, enolase and NRS antisera (image magnification: 1000x).

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Virulence

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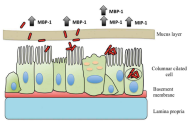

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RESEARCH PAPER

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Comparison of virulence between *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* using *Galleria mellonella* as a host model

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Keywords: adhesion, adhesin, gp43, *Galleria mellonella*, hemocyte, *Paracoccidioides* spp, virulence

Paracoccidioidomycosis is a systemic mycosis, endemic in Latin America. The etiologic agents of this mycosis are composed of 2 species: *Paracoccidioides brasiliensis* and *P. lutzii*. Murine animal models are the gold standard for in vivo studies; however, ethical, economical and logistical considerations limit their use. *Galleria mellonella* is a suitable model for in vivo studies of fungal infections. In this study, we compared the virulence of *P. brasiliensis* and *P. lutzii* in *G. mellonella* model. The deaths of larvae infected with *P. brasiliensis* or *P. lutzii* were similar, and both species were able to reduce the number of hemocytes, which were estimated by microscopy and flow cytometer. Additionally, the phagocytosis percentage was similar for both species, but when we analyze hemocyte-*Paracoccidioides* spp. interaction using flow cytometer, *P. lutzii* showed higher interactions with hemocytes. The gene expression of gp43 as well as this protein was higher for *P. lutzii*, and this expression may contribute to a greater adherence to hemocytes. These results helped us evaluate the behavior of *Paracoccidioides* spp in *G. mellonella*, which is a convenient model for investigating the host-*Paracoccidioides* spp. interaction.

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Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis that is caused by thermally dimorphic fungi from the genus *Paracoccidioides*. This infection is endemic in Latin America and has higher incidences in Brazil, Argentina, Colombia and Venezuela.[1] In Brazil, the annual incidence reaches 30 per million inhabitants, and the mortality rate is 1.4 per million per year.[2] This mycosis has many clinical presentations, and retrospective studies have demonstrated that some factors, such as the host's immune response, age, gender and ethnicity, may influence the clinical presentation.[3] The incidence of chronic infection in the lungs and the upper respiratory tract is more frequent in men older than 30. The sub-acute form is commonly found in children and young adults and in afro-descendant women older than 20 years.[3,4] The *Paracoccidioides* spp mycelial form is found in nature and its propagules are the infective forms of the disease. The infection occurs upon inhalation of the propagules, and during this process, the fungus converts into a yeast form. The temperature is essential for this transformation.[1,5]

Genetic studies have revealed the existence of 3 different phylogenetic species in *P. brasiliensis* called S1, PS2 and PS3.[5] A new species was detected by using molecular approaches; the species started as Pb01-like and has been renamed *P. lutzii* .[6,7] In Brazil, a higher incidence of *P. brasiliensis* is found in the south and southeast region and *P. lutzii* predominantly occurs in the western-central part of Brazil.[8]

The *Paracoccidioides* spp. virulence mechanism involves dimorphism,[9] metalloproteins,[10] adhesins, biofilm formation,[11,12] and melanin production,[13] which play important roles in the infection. Fungal-cell interaction is an essential factor in the establishment of the infection, and for this action, the fungus recognizes specific ligands (carbohydrates and proteins) found in the extracellular matrix, thereby initiating infection and its subsequent spread.[14] In *Paracoccidioides* spp, many proteins known as adhesins mediate this process,[11] and those with multiple functions are known as 'moonlighting' proteins.[15]

Murine models are considered to be the gold standard for the study of fungal pathogenesis and for analyzing the efficacy of antifungal treatments. In 1959, initial studies were conducted in which mice were used as infection models for paracoccidioidomycosis.[16] At present, mice are the standard models for mimicking what occurs in the human disease. Many groups use infection by inhalation routes; however, some studies have described a lack of reproducibility caused by the difficulties the yeast have in reaching the lungs.[17-19] Another important aspect to consider is long-term survival curves when using mice as models; specifically, *P. brasiliensis* (Pb18) could require 2 to 8 months for each

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experiment.[20] Together with economical, logistical and ethical considerations, these challenges limit the use of mammals in infection experiments.[21,22] In this context, the use of alternative animal models or "non-vertebrate mini-hosts" such as *Galleria mellonella* appears to be an option for studying fungal or bacterial disease.[23-26]

The *Galleria mellonella* model is inexpensive to purchase and does not require specialized facilities for maintenance. The relatively large size of the larvae facilitates both the easy handling and injection of defined inoculums and the use of a large number of samples. Furthermore, in contrast to other invertebrate hosts, *G. mellonella* larvae can be maintained at temperatures of 37°C, which is equivalent to the temperature of mammalian hosts. This model presents structural and functional similarities to the innate immune response of mammals. Additionally, the *G. mellonella* innate immune system has 6 types of cells, including phagocytic cells, and the hemocyte density reflects the pathogenicity of the fungi.[21,27,28] *Galleria mellonella* also possesses genes for pathogen-associated molecular pattern recognition, such as toll-like receptors and the transcription factor nuclear factor-κB, in addition to antimicrobial peptides and proteins, most from the families moricin-like protein genes.[29]

The study of the virulence of different fungi such as *Cryptococcus neoformans*,[30,31] *Fusarium* spp,[32] *Candida* spp,[33,34] *Histoplasma capsulatum* and *P. lutzii*.[35] has been performed in *G. mellonella* successfully. *P. lutzii* was able to kill *G. mellonella*, however was not possible to establish a correlation between cell concentration and larvae death. Besides that, the infection caused larvae melanization and granuloma-like formation in a yeast concentration-dependent manner.[35] However, this model was only evaluated in *P. lutzii* but not in *P. brasiliensis*. The discovery of the *P. lutzii* species leads to new questions about virulence, clinical manifestations and diagnosis.[36,37] Moreover, because of the recent classification of 2 distinct species (*P. brasiliensis* and *P. lutzii*), few studies have compared the virulence and host interactions among them. Given this outlook, the aim of this study was to evaluate the virulence of *P. brasiliensis* and compare it with *P. lutzii* in the *G. mellonella* model. In addition, the hemocyte response to the infection was determined by measuring the hemocytic density, phagocytosis, hemocyte-fungal interactions as well as adhesins genes enolase, gp43, 14-3-3, malate synthase, and triosephosphate isomerase.

Results

Virulence of *P. brasiliensis* and *P. lutzii* in *G. mellonella*

This study provides the first description of a *P. brasiliensis* and *P. lutzii* virulence comparison in *G. mellonella*. The larvae were infected with 3 different concentrations of both species (5×10^5, 1×10^6 and 5×10^6 cells/larva) with the objective of finding the concentration that was able to kill them within 7 days of starting the experiment (**Fig. 1**). This time is considered appropriate for the use of the *G. mellonella* model because of the length of the insect's life cycle. The 1×10^6 cells/larva concentration of *P. brasiliensis* was able to kill more larvae than the same concentration for *P. lutzii* but in the end of the experiment almost 60% of the larvae were alive. The 5×10^6 cells/larva concentration was selected because the majority of larvae were dead in within the observation period. An analysis of the survival curve for both *Paracoccidioides* species, for the 5×10^6 cells/larva concentration, showed that the time necessary to reach 50% killing ($TD_{50\%}$) ranged from 3 to 4 days for *P. brasiliensis*, and the $TD_{50\%}$ ranged from 4 to 5 days for *P. lutzii*. However, the survival curve profiles were very similar, which suggested that *P. brasiliensis* and *P. lutzii* have similar virulence levels ($p = 0.3051$) in this model.

Histopathology analysis

The histopathology of uninfected and infected *Paracoccidioides* spp. larvae was also performed to study the infection by this yeast. Larvae were collected after one hour and 4 days of infection and stained with periodic acid Schiff (PAS). After one hour of infection, *P. brasiliensis* (**Fig. 2C**) and *P. lutzii* (**Fig. 2D**) were visualized in the periphery of the *G. mellonella* tissues. After 4 days of infection, *G. mellonella* tissues of larvae infected with *P. brasiliensis* (**Fig. 2E**) and *P. lutzii* (**Fig. 2F**) were destroyed when compared with uninfected larvae (**Fig. 2A, B**), and both fungal species were able to multiply inside the larvae. The cellular response to the invasion, encapsulation and granuloma, in addition to the humoral phenomenon, that is, the melanization, were observed in the histopathology (**Fig. 3**).

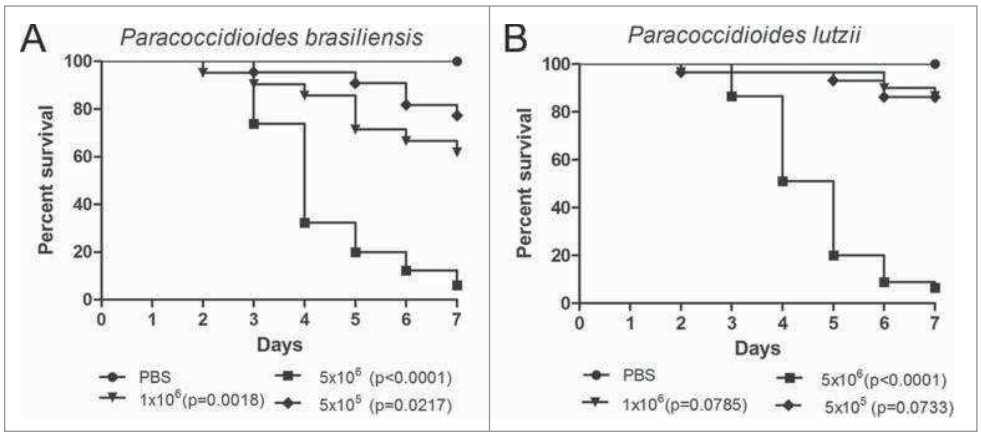

Figure 1. Survival curves of *G. mellonella* infected with *P. brasiliensis* (**A**) and *P. lutzii* (**B**) at different concentrations; PBS-infected larvae were used as controls and statistical significance ($p < 0.05$) is relative to the PBS control.

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Hemocyte density after infection by *Paracoccidioides* spp

Changes in the hemocyte density are one important factor to observe in the larvae response when analyzing virulence. Larvae were infected with 5×10^6 cells/larvae and incubated at 37°C for one and 3 hours and measure the hemocyte density of *G. mellonella* after these periods of infection by *Paracoccidioides* species by using 2 methodologies, namely microscopy and flow cytometry (**Fig. 4**). For microscope estimation was used a hemocytometer under a brightfield microscope. According to the microscopy analyses, there was a hemocyte reduction of 5 and 8 times in both species at 1 and 3 h after infection, respectively (**Fig. 4A**). In the flow cytometry analyses, the hemocyte density after infection with *P. brasiliensis* or *P. lutzii* decreased 3 times for both incubation times (**Fig. 4B**). The results indicate that infections with 5×10^6 cells/larva of *P. brasiliensis* and *P. lutzii* reduced the density of hemocytes compared with the non-infected larvae during the analyzed time points. Additionally, the hemocyte density in the larvae infected with *P. brasiliensis* was similar to that with *P. lutzii*.

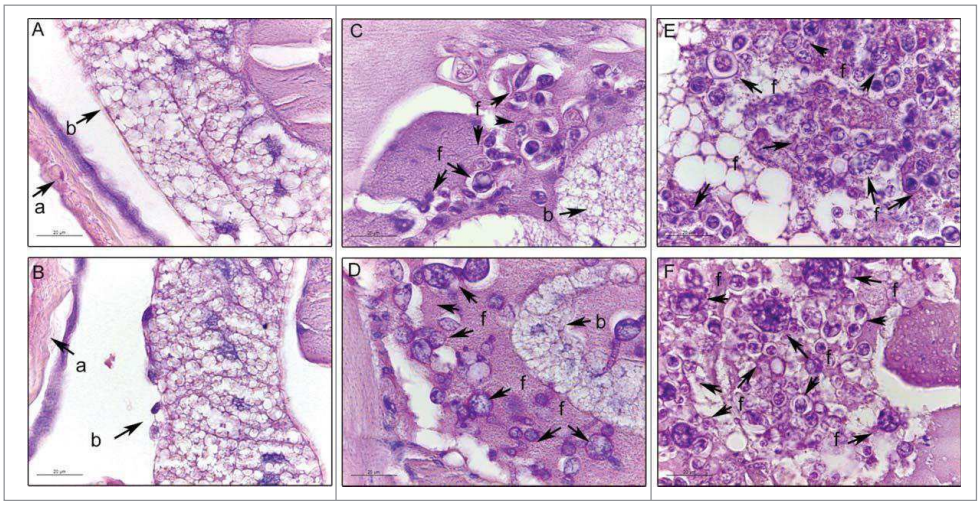

Figure 2. Histology of *G. mellonella* stained with PAS. Uninfected larvae (**A, B**); larva infected with *P. brasiliensis* after 1 hour (**C**) and after 4 days (**E**); larva infected with *P. lutzii* after 1 hour (**D**) and after 4 days (**F**). Amplification 1000x. Arrows indicates *P. brasiliensis* or *P. lutzii*. Structures annotated: (a) cuticle; (b) adipose bodies; (f) fungal cells.

As described above, hemocyte estimation was evaluated by using a traditional hemocytometer under a brightfield microscope and compared with the flow cytometer results. For this, *Paracoccidioides* spp suspension of 5×10^8 cells/mL was stained with 100 μM CFDA-SE for 30 minutes at 37°C and hemocytes samples of infected and uninfected larvae were stained with 0.165 μM Alexa Fluor® 647 phalloidin conjugates. The development of the flow cytometry assay for hemocyte estimation provide faster results and the possibility of analyze a greater amount of samples, moreover, because of the double stain the differentiation of fungi or hemocyte cells are precise. Both methodologies were comparable by Pearson coefficient (**Fig. 5**). Correlation coefficients for the hemocyte density in *G. mellonella* that were injected with PBS were 0.83 at 1 h ($p=0.0002$) and 0.80 at 3 h ($p=0.0006$), and those injected with *P. brasiliensis* were 0.86 for 1 h ($p<0.0001$) and 0.95 at 3 h ($p<0.0001$); those injected with *P. lutzii* were 0.96 for 1 h ($p<0.0001$) and 0.90 at 3 h ($p<0.0001$). This result suggested that a good correlation was found between the hemocyte density obtained by flow cytometry and by the standard method of counting cells on a hemocytometer (microscopy) in larvae that were infected with PBS or *Paracoccidioides* species at the different time points.

Paracoccidioides spp. - hemocyte interactions

The hemocyte-fungal interactions were evaluated by flow cytometry methodology. *Paracoccidioides* spp suspension of 5×10^8 cells/mL was stained with 100 μM CFDA-SE as described above and larvae were infected. After 1 and 3 h of infection, hemocytes samples of infected and uninfected larvae were stained with 0.165 μM Alexa Fluor® 647 phalloidin conjugates. The double-stained population (the CFDA-SE-positive and phalloidin-positive population) was examined for its hemocyte-fungal interactions using flow cytometry, and the percentage

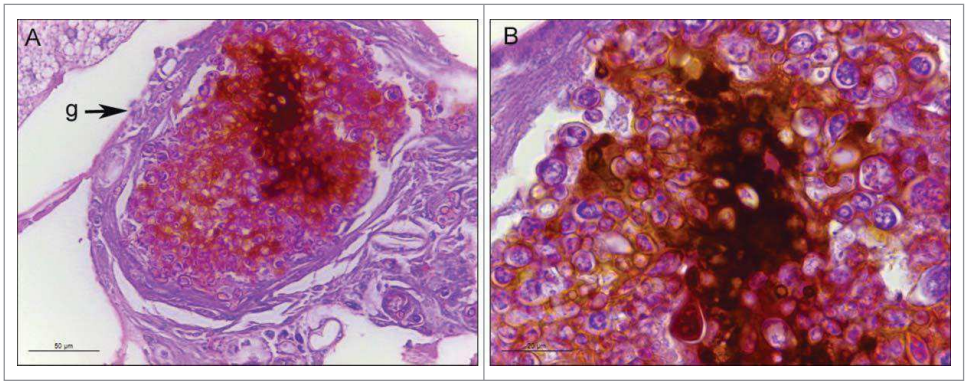

Figure 3. The cellular and humoral response to infection with *P. brasiliensis* after 3 days. Granuloma structure, amplification 400X (**A**); melanization and encapsulation process, amplification 1000x. (**B**). Similar structures were observed during *P. lutzii* infection. Structure annotated: (g) granuloma-like structure.

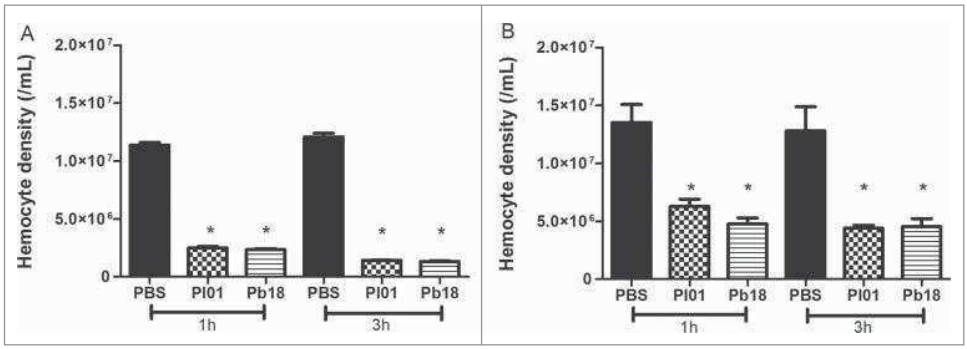

Figure 4. Hemocyte density, as obtained by microscopy (**A**) and flow cytometry (**B**), in *G. mellonella* larvae infected with *P. brasiliensis* (Pb18) and *P. lutzii* (PI01) when assessed after 1 and 3 h. The asterisks indicate statistical significance ($p < 0.05$) relative to the PBS control.

of double-stained cells in the population is shown in **Figure 6**. An important difference was observed in the hemocyte-fungal interaction profile between the fungal species. The *P. brasiliensis*-hemocyte interactions were 13 % and 14 % after 1 and 3 h, respectively (**Fig. 6A**); however, the *P. lutzii* interaction was 49 % and 48 % after 1 and 3 h, respectively (**Fig. 6B**). To evaluate the efficiency of the innate immune system against these species and consequentially the ability to escape from them, hemocytes phagocytosis was evaluated. The *Paracoccidioides* species were stained with 10 μg/mL Calcofluor white and larvae were infected with stained cells at 5×10^6 cells/larvae and incubated for 3 h at 37°C. After the infection hemocytes samples of infected larvae were stained with 0.165 μM Alexa Fluor→ 647 phalloidin conjugates and images were taken and a percentage of hemocytes containing yeasts was calculated using fluorescence microscopy. In this assay, 5 % of the hemocyte cells from *G. mellonella* were able to phagocytize *P. brasiliensis* or *P. lutzii* (**Fig. 7**), these results demonstrated that innate immune system of *G. mellonella* recognize equally these species.

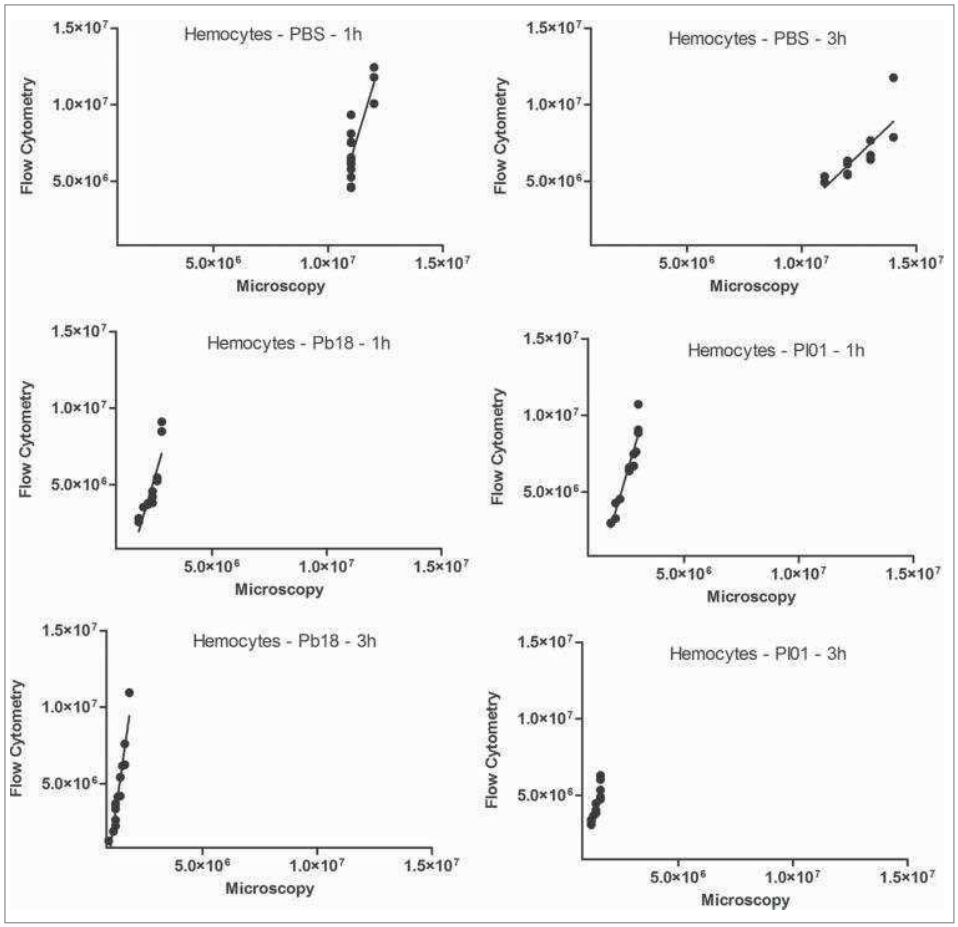

Figure 5. Linear regression and scatter plots for hemocyte counts (/mL) obtained by flow cytometry and microscopy (manually) in *G. mellonella* larvae injected with PBS, *P. brasiliensis* (Pb18 - 5×10^6 cells/larva) or *P. lutzii* (PI01 - 5×10^6 cells/larva) that were assessed after 1 and 3 h.

Paracoccidioides spp. adhesin gene expression

Adhesion is an important factor in establishing an infection. The gene expression of important adhesins in *Paracoccidioides* spp, namely enolase, gp43, 14-3-3, malate synthase, and triosephosphate isomerase, was analyzed after the infection in *G. mellonella.* For this, after one hour of infection the hemolymph from infected and uninfected larvae was collected and *Paracoccidioides* spp. cells were recovered by centrifugation, RNA was extracted and real-time PCR was performed. The *P. lutzii* expression of gp43 and triosephosphate isomerase were higher than they were in *P. brasiliensis* (13-folds ($p = 0.0011$) and 2.5-folds ($p = 0.0018$), respectively). This higher gene expression could explain the potential adhesion by *P. lutzii* to the hemocytes. For enolase, 14-3-3 and malate synthase, the expressions for both species were similar (**Fig. 8**).

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Gp43 importance during *Paracoccidioides* spp- hemocyte interaction

Because of the importance of gp43 in the pathogenicity of *Paracoccidioides* spp, a Western blot assay was performed to verify the presence of this adhesin and compare it with the gene expression results. For this purpose, larvae protein extracts were obtained 3 days after infection with *P. brasiliensis*, or *P. lutzii* and control proteins were collected from *Paracoccidioides* spp., uninfected *G. mellonella*, and purified gp43. The presence of a 43 kDa fraction was observed in samples taken from *G. mellonella* that had been infected with *P. lutzii* and *P. brasiliensis* (**Fig. 9**). The antibody used in this work was obtained against the *Paracoccidioides* cells, and thus the test could detect other proteins in addition to gp43. In extracts from uninfected *G. mellonella* the presence of this protein was not detected.

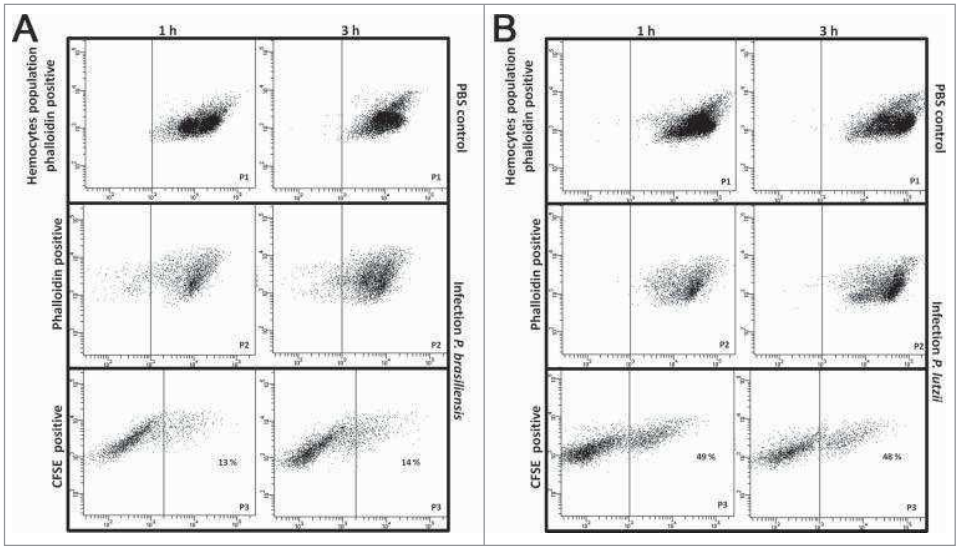

Figure 6. Hemocyte-fungal interaction obtained by flow cytometry after infection with *P. brasiliensis* (**A**) and *P. lutzii* (**B**). In gate P1, the hemocyte population is phalloidin positive with non-infected larvae; in gate P2, the hemocyte population is phalloidin-positive with infected larvae; and gate P3 holds the doubly stained population (hemocyte-stained phalloidin and fungal-stained CFDA-SE) that are considered hemocyte-fungal interactions that were obtained by flow cytometry after infection with *P. brasiliensis* (**A**) and *P. lutzii* (**B**). Differences in the hemocyte-fungal interaction between the fungal species ($p < 0.05$).

Discussion

Ethical questions are restricting the use of experimental vertebrate animals.[38] In this context, the use of alternative animal models such as amoebae (*Acanthamoeba castellanii* and *Dictyostelium discoideum*), nematodes (*Caenorhabditis elegans*), insects (*Drosophila melanogaster* and *Galleria mellonella*) and recently embryonated chicken eggs has emerged in recent years, and they provide good alternatives for studying fungal pathogenesis.[39-43]

In the last decade, *G. mellonella* has become more accepted as an alternative animal model of infection.[22,44] The structural and functional similarities between the immune responses of insects and mammals permits the insects to be used to predict the likely response of mammals to a variety of pathogenic microorganisms.[45] The hemolymph has analogous functions to blood and contains cells called hemocytes. There are at least 6 types of hemocytes and plasmatocytes, and granulocytes are the most abundant phagocytic cell types. Furthermore, antimicrobial peptides are also responsible for killing invading microorganisms.[46,47]

In this study, we used *G. mellonella* to establish a comparison between the virulence of 2 *Paracoccidioides* species. The survival curve profiles show that *Paracoccidioides* spp caused the concentration-dependent death of *G. mellonella*. Likewise, a correlation between the yeast cell number of *P. lutzii* and the time to death of *G. mellonella* was observed in a previous study, although the tested concentrations (10^1 to 10^6 cells/larva) were different from those in the present study.[35] However, the death time of larvae infected with 10^6 yeast cells was lower than that in our study. One explanation for this finding may be related to the different preparations of the inoculums. The cells of the fungus are very irregular in size and shape, and we believe that the use of inoculums with standard cell sizes was fundamental for the experiment.

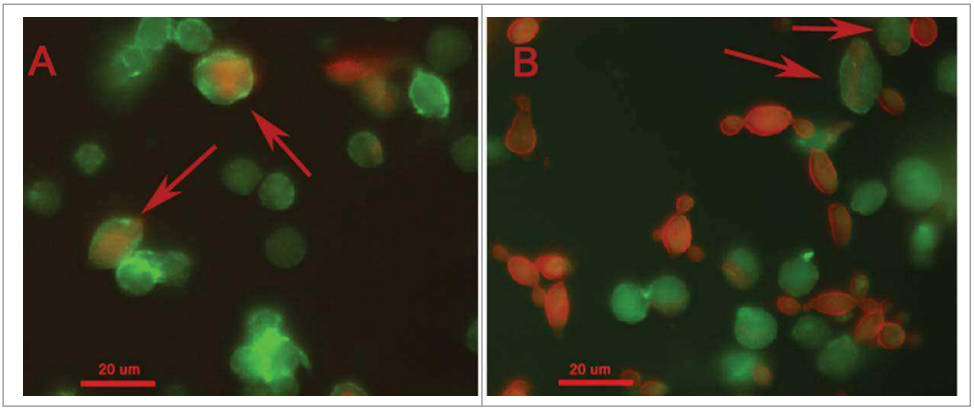

Figure 7. Phagocytosis of *Paracoccidioides* spp by hemocytic cells after 3 h of infection with 5×10^6 cells/larva from *P. brasiliensis* (**A**) and *P. lutzii* (**B**). The arrow indicates the phagocytosis.

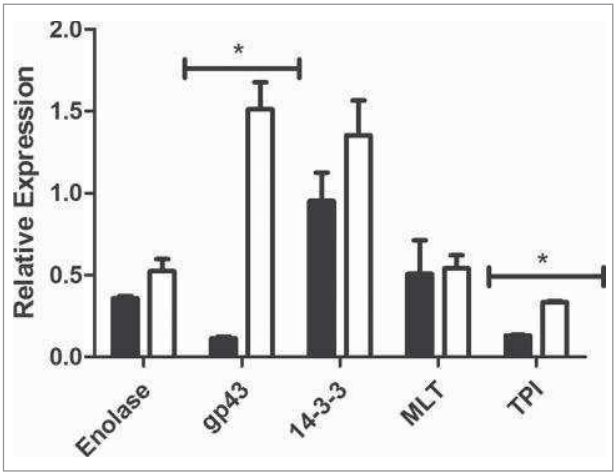

Figure 8. Relative gene expression of enolase, gp43, 14-3-3, triosephosphate isomerase and malate synthase in *P. brasiliensis* (black) and *P. lutzii* (white), (*) $p < 0.05$.

Paracoccidioides brasiliensis caused larval death faster (3-4 days) than *P. lutzii* (4-5 days). Furthermore, the histopathology of infected larvae at 1 hour and 4 days after the infection showed

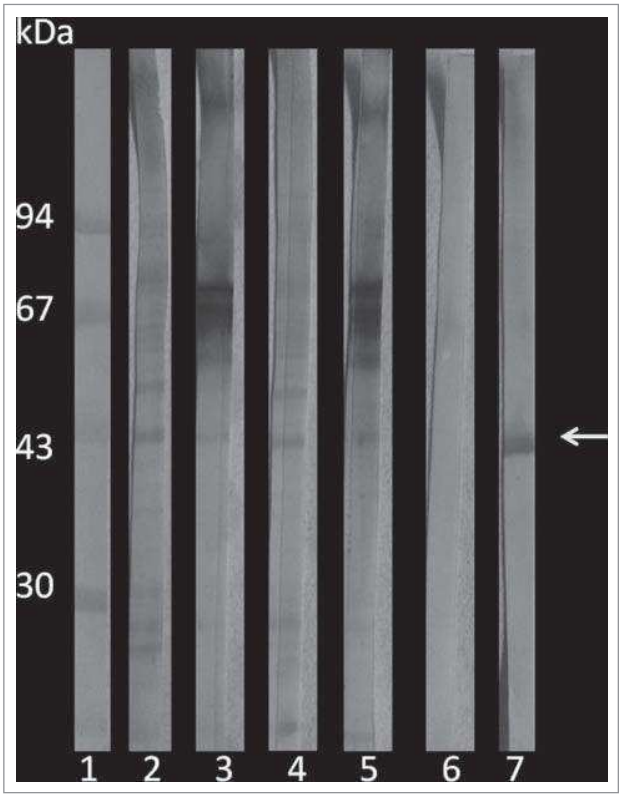

Figure 9. Western blot of *G. mellonella* infected with *P. brasiliensis* or *P. lutzii*. 1) molecular weight marker; 2) *P. lutzii*; 3) *G. mellonella* infected with *P. lutzii*; 4) *P. brasiliensis*; 5) *G. mellonella* infected with *P. brasiliensis*; 6) *G. mellonella* extract infection; and 7) purified gp43. The arrow indicates the gp43.

that both species were able to multiply inside the larvae and destroy the *G. mellonella* tissue. Moreover, encapsulation, melanization and granuloma-like structures were observed in the histopathology. The presence of granulomas were described during the paracoccidioidomycosis in murine model affecting the lung and spleen.[48] The effect of melanization on the elimination of pathogens is widely discussed, and the activation of the pro-phenol oxidase cascade produces substances that are very toxic to microorganisms and also helps in phagocytosis, aiding in the survival of invertebrates.[49] The encapsulation occurred when the cells were unable to phagocytize and thus tried to control the invasion by creating a cell barrier.[50]

By using 2 techniques, we demonstrated that the infection of *Paracoccidioides* spp. causes a decrease in the hemocyte density. After just one hour of infection, a significant reduction in the hemocyte density was observed in the infection with *P. brasiliensis* or *P. lutzii* compared with the control. The same reduction was observed after 2 more hours of incubation. Moreover, no significant difference was observed between the 2 tested species. Numerical differences between the techniques (flow cytometry and hemocytometer count) were observed; however, the hemocyte reduction profile remained the same. The hemocyte density is an important factor for evaluating virulence because low survival after infection is related to low hemocyte density.[51] In addition, the hemocyte density can vary during the infection, and a decrease was observed during the first hours of the infection.[52]

Another important aspect of this work is the standardization of hemocyte counting. We developed a practical flow cytometry protocol with double staining to determine the hemocyte density and the percentage of hemocyte-fungal interaction. The statistical analyses show a good correlation between the traditional hemocytometer estimation and the results obtained by flow cytometry. Thus, flow cytometry may be considered a reliable method for hemocyte counting because it is faster, more practical and gives precise results.

Phagocytosis was evaluated during the early stages of infection (3h), for both species, 5% phagocytosis was found, however, *P. brasiliensis* showed lower interactions with hemocytes when compared with the *P. lutzii* infection, which would indicate a better escape mechanism by this species from phagocytosis. The choice for 3h of post-infection time for phagocytosis assay is because of the significant reduction of hemocyte density, phagocytosis could be difficult to observe after more hours of infection. Other studies have demonstrated in *vitro* phagocytosis of *P. brasiliensis* around 7% after 6 h of infection and 6-12% after 24 h of infection depending on the type of macrophage studied.[53,54] Even though, in this model we have similar phagocytosis index. Additionally, the literature has been shown the absence of digestive and killing capacities of PMNs from paracoccidioidomycosis patients (susceptible individuals) in unambiguous contrast of cells from healthy (resistant) individuals.[55]

Studies showed significant similarity between the rates of *in vitro* phagocytosis of opsonized *C. albicans* by neutrophils and hemocytes. In the same way that it occurs for neutrophils, phagocytosis by hemocytes also stimulates the respiratory burst pathway involving NADPH oxidase activity and superoxide

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production for efficient microbial killing.[56] Other similarities between neutrophils and hemocytes were demonstrated by treating the hemocytes with cytochalasin b and nocodazole, leading to an increase in the susceptibility of the *G. mellonella* larvae to the infection caused by a disruption of hemocyte function, which is the same way that they affect mammalian neutrophils. Earlier studies have described noticeable neutrophil infiltrates in *Paracoccidioides* lesions of experimental animal models and also in tissue samples from patients. Neutrophils were present in *P. brasiliensis* microabscesses and in loose granulomas from oral lesions.[57] Nevertheless, they are usually not sufficient to eliminate the fungus cells completely.

The hemocyte fungal interaction was studied in this work as an important key to discuss the difference between the virulence of both fungi. Differences in the virulence between *Paracoccidioides* spp strains have been reported, and they are likely related to antigenic differences and the adhesion process.[58,59] In this study, we observe that *P. lutzii* is able to interact with hemocytes 3.7-folds more than *P. brasiliensis.*

Different proteins with roles in fungal-cell adhesion are well-described for *P. brasiliensis*, and most of them participate in the glycolytic pathway, tricarboxylic acid cycle and glyoxylate cycle, and because of their multiple functions, these proteins are known as 'moonlighting' proteins.[15] In this work, we analyze the gene expression of 5 important adhesins from *Paracoccidioides* spp, namely enolase, gp43, 14-3-3, triosephosphate isomerase and malate synthase. A significant difference of expression for gp43 and triosephosphate isomerase was observed in *P. lutzii* after the infection of *G. mellonella*. Because of the importance of gp43, we investigated its expression by Western blot assay during the *G. mellonella* infection, and we tested if its presence can explain the higher hemocyte adhesion found for this species. Although gp43 production is detected *in vitro* within less than one hour of incubation,[60] we use 3 days of infection to detect this protein because of the low amount of yeast present in the sample. The 43 kDa glycoprotein was the first to be described as an adhesin and to be able to bind to laminin,[61,62] and it was more recently shown to bind both fibronectin and laminin. In *Paracoccidioides* spp., certain additional adhesins have also been described and may play an important role in pathogenesis.[59,63-67]

The importance of gp43 was studied.[68] by silencing the PbGP43 gene. No morphology, vitality or fungal growth was affected by the reduction in expression of gp43 gene. However, the expression of this protein was reduced *in vitro* and thus affected the recovery of yeasts from macrophages that were adhered to or phagocytized; and it was reduced *in vivo* from the lungs of infected mice. In addition to being described as an adhesin, the 43 kDa glycoprotein is the primary diagnostic antigen of *P. brasiliensis*.[69] Recent studies have demonstrated that gp43 is highly specific for PCM caused by *P. brasiliensis* and false negative for *P. lutzii*.[70,71] By analyzing the peptide sequence of gp43 protein in both species, an 81 % similarity was found; however, the molecular models show differences between them. Specifically, the N-glycosylation site is absent, and the glucanase site and its enzymatic activity are preserved in gp43 from *P. lutzii*.[36] The *Galleria* model has the same behavior as the *in vivo* model, and gp43 from *P. lutzii* may be expressed in the same way as it is in human disease. Moreover, gp43 appears to play a role in adhesion processes during the first hours of infection.

Triosephosphate isomerase (TPI) is a glycolytic enzyme that is present in the cytoplasm, and it is responsible for the conversion of dihydroxy acetone phosphate to glyceraldehyde-3-phosphate. Its gene was also more expressed in the *G. mellonella P. lutzii* infection. The importance of triosephosphate isomerase in paracoccidioidomycosis was first described when it was determined to be a protein that can react with the sera of PCM patients.[72] Moreover, when *P. lutzii* cells were treated with anti-TPI polyclonal antibody, an inhibition of the interaction with epithelial cells was observed. TPI plays an important role in host cell binding and could be considered an adhesion.[66] Triosephosphate isomerase is also one of the molecules responsible for interactions between other microorganisms. This binding may occur between the TPI of *S. aureus* and the mannotriose present in the capsule of *C. neoformans*, and the consequence of the binding is the death of *C. neoformans*.[73] Both microorganisms could be found in the nasopharynx, with *S. aureus* in healthy individuals. The hypothesis is that the presence of *S. aureus* in human nasal cavities may prevent the entry of *C. neoformans*,[74] and immunoelectron microscopy studies confirm that this cytoplasmic enzyme was localized on the cell surface and interacted with the mannotriose of the *C. neoformans*.[75] More studies are necessary to explain the differences between both species in the *G. mellonella* model as well as in human infections.

When studying virulence, it is necessary to consider that multifactorial events belong to the host and to the microbe; moreover, the likely phenomena of latency, colonization, commensalism and death could occur during host-microbe interactions.[76,77] In this study, we evaluated the virulence of *P. brasiliensis* and *P. lutzii* in a *G. mellonella* model by using survival curves, hemocyte density, phagocytosis, hemocyte interaction with *Paracoccidioides* spp yeast cells and adhesin expression. The parameter that differentiates the virulence of both species was yeast-hemocyte interaction, in which we found that *P. lutzii* has better abilities to adhere to the hemocytes than *P. brasiliensis*, and we attributed this capacity to the higher expression of important adhesins (gp43 and triosephosphate isomerase). However, some studies have addressed the expression of gp43 during *P. lutzii* infection in humans,[36] and in *G. mellonella*, it seems to be important to the adhesion process. These results were very important to understand the differences and similarities in both species better. Furthermore, *G. mellonella* was demonstrated to be a suitable model to study this fungus because of the ethical issues, cost, easy rearing and manipulation of larvae and reduced time, and the parameters analyzed in this study could be useful in the evaluation of different *in vivo* assays.

Material and Methods

Strains and growth conditions

Paracoccidioides brasiliensis (São Paulo, Brazil) (Pb18) and *P. lutzii* Pb01-like strain 01 (ATCC MYA-826/ Goiania, Brazil)

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(Pl01) in the yeast phase were used. Both species were maintained in Fava-Netto medium at 37°C and subcultured every 4-5 days. After this period, the fungal mass was transferred to a Brain Heart Infusion (BHI) broth supplemented with 1 % glucose (BHI suppl).[78] for 3-4 days at 37°C at 150 RPM to obtain exponential-phase cells.[79,80] The *Paracoccidioides* spp strain stock was recently reisolated from cell culture.

Inoculum standardization

The *Paracoccidioides* spp growths were washed 3 times with PBS buffer after their cell clumps were separated by repeated passages through an 18-gauge needle (Becton Dickinson, Brazil) into a 5 mL syringe (Becton Dickinson, Brazil). The suspension was filtered through 40 μm nylon cell strainers (Corning, USA) to obtain cells of homogeneous sizes. The cell concentrations were estimated by using a hemocytometer. The inoculum was prepared in PBS plus 20 mg/L of ampicillin to prevent bacterial contamination.

***Galleria mellonella* rearing and larvae manipulation**

The larvae were fed with wax and pollen and maintained at 25°C.[81] until reaching 150-200 mg in weight. Larvae without color alterations and with adequate weights were selected, placed in Petri plates and incubated at 37°C overnight under protection from light. The insect pro-leg regions were cleaned with 70% ethanol before the experiments. Using a syringe (Hamilton, USA), 10 μL of inoculum was injected through the last left pro-leg of the larvae. Each experiment was repeated at least 3 times.

Survival assay

Sixteen larvae per group were inoculated with different inoculum concentrations of *Paracoccidioides* spp. (5×10^5, 1×10^6 and 5×10^6 cells/larva) for the survival assay. The control groups were larvae that were injected with PBS and untouched larvae. The larvae were incubated at 37°C, and this death was monitored daily for 7 days by visual inspection, which was done by checking for a lack of movement after touching the larvae with forceps.

Histopathology analysis

Uninfected and infected larvae were collected after one hour and 4 days of infection and processed as described by Scorzoni el al, 2013.[82] for a periodic acid Schiff (PAS) stain. Three tissue sections were observed per larva with a Leica DMI3000 microscope.

Determination of hemocyte density by flow cytometry

A *Paracoccidioides* spp suspension of 5×10^8 cells/mL was stained with 100 μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE/Sigma Aldrich, USA) for 30 minutes at 37°C and washed 3 times with PBS at 5000 rpm for 5 minutes. *Galleria mellonella* larvae were infected with the previously stained suspension and incubated at 37°C for one and 3 hours. A control group was set up by inoculating PBS. After the infection period, hemolymph samples (10 μL) were collected and stained with 50 μL of 0.165 μM Alexa Fluor® 647 phalloidin conjugates (Invitrogen) diluted in cold 4 % paraformaldehyde (Sigma Aldrich, USA) plus 0.25 % Triton X-100 (Amersham Biosciences, USA) and incubated for 20 minutes. The hemocyte density was determined by flow cytometry (BD FACSCanto) by acquiring 60 seconds on medium acquisition mode. In this mode, 60 μL was acquired, and the correlation was made in milliliters. A dot plot SSC-A (side scatter) by phalloidin (APC) was made to analyze the hemocyte number in control PBS (gate P1) and after the infection (gate P2). The hemocytes from larvae injected with PBS were compared with the infected ones.

Determination of hemocyte density using a hemocytometer

Larvae were infected with 5×10^6 cells/larvae and incubated at 37°C for one and 3 hours. After the infection period, hemolymph samples were collected and diluted at 1:20. A control group was set up by inoculating with PBS. The hemocyte density was estimated by using a hemocytometer under a brightfield microscope. The control was compared with PBS injected larvae.

Hemocyte-fungal interaction assay by flow cytometry

The hemocyte-fungal interactions were evaluated by flow cytometry methodology. Double-positive fluorescent-stained cells (with phalloidin and CFDA-SE) were used to observe fungal cells that adhered to or phagocytosed the hemocytes. Stain control was used on the fungal cells and hemocytes alone, under unlabeled and labeled conditions, with both stains and one stain alone. All cytometry data were analyzed using the BD FACS Diva software.

Phagocytosis assay

The yeast cells were stained with 10 μg/mL Calcofluor white (Sigma Aldrich, USA) for 30 min at 37°C. After that, 10 larvae were infected with stained cells at 5×10^6 cells/larvae and incubated for 3 h at 37°C. The hemolymph samples were collected and the hemocytes were stained as described for the flow cytometry assay. The hemolymph samples of each larva were placed in a 96-well plate (made of a plate glass bottom and black borders; Glass Bottom/GE), and the images were acquired with In Cell Analyzer 2000-GE at an amplification of 60x. One hundred hemocytes from each larva were counted and the percentage of hemocytes containing yeasts was calculated.

***Paracoccidioides* spp gene expression of adhesins by real-time PCR**

Genes encoding for enolase, gp43, 14-3-3, triosephosphate isomerase and a malate synthase protein described as adhesin in *Paracoccidioides* spp. were analyzed by real-time PCR. For this analysis, 60 larvae were infected with 5×10^6 cells/larvae of *P. brasiliensis* or *P. lutzii* and incubated for one hour at 37°C, and uninfected groups of larvae and *P. brasiliensis* or *P. lutzii* without pass-through infection were used as controls. The hemolymph from each group was collected, and the *Paracoccidioides* spp cells were recovered by centrifugation and washed twice with PBS. RNA was extracted with RNeasy (Qiagen). First-strand cDNA synthesis was performed with reverse transcriptase (RevertAidTM H Minus Reverse Transcriptase, Fermentas, Canada). Real-time PCR was performed with Maxima® SYBR Green/ROX qPCR

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Master Mix (2X) (Fermentas, Canada) using Applied Biosystems 7500 cycler equipment. The relative expression was calculated by using $2^{-\Delta\Delta CT}$ values with 60S ribosomal L34 as the housekeeping gene; this constitutive gene is widely described as a control in different studies on paracoccidioidomycosis.[83,84]

Protein Extraction and Western Blot assay

To perform a Western blot, 16 *G. mellonella* larvae at 3 days of infection were used with *Paracoccidioides* spp., along with 16 larvae that were not infected and *Paracoccidioides* cells. The protein extraction was performed first, which involved adding 3 mL of 10 mM Tris-HCl pH 6.8 and protease inhibitor mix to each sample. The samples were then lysed with glass beads and vortexed. The material was centrifuged at 5000 rpm for 1 hour at 4°C. After centrifugation, the supernatant was collected and the proteins were precipitated with 10 % TCA in 90 % acetone. The precipitated proteins were diluted in 10 mM Tris-HCl pH 6.8 and quantified by Bradford assay. One hundred micrograms of each sample was applied to an SDS-PAGE gel to separate the proteins. The proteins were subsequently transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody that was obtained against a total *P. brasiliensis* extract [85] and the secondary antibody was rabbit anti-mouse IgG conjugated to peroxidase. The reaction was developed with chromogen: diaminobenzidine (DAB) substrate diluted in PBS that was added to hydrogen peroxide. For controls, we used the protein extract of uninfected *G. mellonella*, purified gp43 and *Paracoccidioides* spp protein.

Statistical Analysis

Graphs and statistical analyses were performed with GraphPad Prism 5 (La Jolla CA, USA). Survival curves were analyzed by Log-rank (Mantel-Cox) test. The hemocyte density data were analyzed by ANOVA with Tukey's post-test. A comparison of the hemocyte density by using a flow cytometry and a hemocytometer was performed with a Pearson correlation, and the real-time PCR was analyzed by t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Paracoccidioides-host Interaction: An Overview on Recent Advances in the Paracoccidioidomycosis

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Paracoccidioides brasiliensis and *P. lutzii* are etiologic agents of paracoccidioidomycosis (PCM), an important endemic mycosis in Latin America. During its evolution, these fungi have developed characteristics and mechanisms that allow their growth in adverse conditions within their host through which they efficiently cause disease. This process is multi-factorial and involves host–pathogen interactions (adaptation, adhesion, and invasion), as well as fungal virulence and host immune response. In this review, we demonstrated the glycoproteins and polysaccharides network, which composes the cell wall of *Paracoccidioides* spp. These are important for the change of conidia or mycelial (26°C) to parasitic yeast (37°C). The morphological switch, a mechanism for the pathogen to adapt and thrive inside the host, is obligatory for the establishment of the infection and seems to be related to pathogenicity. For these fungi, one of the most important steps during the interaction with the host is the adhesion. Cell surface proteins called adhesins, responsible for the first contact with host cells, contribute to host colonization and invasion by mediating this process. These fungi also present the capacity to form biofilm and through which they may evade the host's immune system. During infection, *Paracoccidioides* spp. can interact with different host cell types and has the ability to modulate the host's adaptive and/or innate immune response. In addition, it participates and interferes in the coagulation system and phenomena like cytoskeletal rearrangement and apoptosis. In recent years, *Paracoccidioides* spp. have had their endemic areas expanding in correlation with the expansion of agriculture. In response, several studies were developed to understand the infection using *in vitro* and *in vivo* systems, including alternative non-mammal models. Moreover, new advances were made in treating these infections using both well-established and new antifungal agents. These included natural and/or derivate synthetic substances as well as vaccines, peptides, and anti-adhesins sera. Because of all the advances in the PCM study, this review has the objective to summarize all of the recent discoveries on *Paracoccidioides*-host interaction, with particular emphasis on fungi surface proteins (molecules that play a fundamental role in the adhesion and/or dissemination of the fungi to host-cells), as well as advances in the treatment of PCM with new and well-established antifungal agents and approaches.

Keywords: ***Paracoccidioides brasiliensis,*** ***Paracoccidioides lutzii*****, fungi–host interaction, paracoccidioidomycosis, *Paracoccidioides* pathogenicity and virulence**

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INTRODUCTION

It is estimated that about 1.2 billion people worldwide suffer from fungal diseases. Some of these are invasive or chronic and difficult to diagnose and treat. It is estimated that 1.5 to 2.000.000 people die of fungal infections each year, surpassing those who die from other causes (Denning and Bromley, 2015). In Latin America, the rich diversity of biomass and climates provides a rich range of habitats for different microorganisms, including these pathogenic fungi responsible for endemic mycoses and that have an important impact on public health: histoplasmosis, coccidioidomycosis, and paracoccidioidomycosis (PCM; Colombo et al., 2011).

The *Paracoccidioides* spp. specie complex is dimorphic fungi that are the etiologic agents of PCM. This is the most important systemic mycosis in Latin America with Brazil, Venezuela, and Argentina being the countries with the greatest number of patients. Non-autochthonous cases have been described outside endemic areas in patients who have lived in or visited Latin America. Brazil, which accounts for 80% of the cases, concentrated the occurrence in its southeastern, southern, and Midwestern regions. PCM is an endemic mycosis that is responsible by the highest cause of mortality and the eighth most important cause of mortality from chronic infectious diseases reaching rates of 1.65 deaths per 106 inhabitants (Coutinho et al., 2002; Bocca et al., 2013). According to Martinez (2010), an estimated 3,360 new cases per year reflect the fatality and mortality rates attributed to PCM in Brazil. Through epidemiological surveys it's known that PCM occurs throughout Brazil (Blotta et al., 1999; Bellissimo-Rodrigues et al., 2011; Loth et al., 2011; de Souza et al., 2014; Vieira et al., 2014). Recent eco-epidemiological studies (**Table 1**) have been demonstrating the occurrence of the PCM in different regions of the Brazil, warning the scientific community about the importance of this disease to the country.

The *Paracoccidioides* genus is composed of thermally dimorphic fungi classified in the *Onygenales* order and in the *Ajellomycetaceae* family (Untereiner et al., 2004). Currently, due to advances in phylogenetic studies of different *Paracoccidiodes* isolates, this genus is divided into two species: *P. brasiliensis* and *P. lutzii*, being the first divided into three different phylogenetic species, S1, PS2, and PS3 (Matute et al., 2006; Carrero et al., 2008; Marini et al., 2010). Agents of systemic mycoses, such as *P. brasiliensis* and *P. lutzii*, express factors that facilitate their survival in severe conditions inside the host cells and tissues, and as such, benefit the disease's development (Casadevall and Pirofski, 1999). The successful colonization of host tissues by the fungus is thus a complex event, usually involving various regulatory mechanisms of cellular homeostasis and the expression of different virulence factors during infection that allows the fungi to cause this systemic mycosis with deep extension in the host organism.

The PCM fungi primarily infects male peasants, between 30 and 60 years of age (Svidzinski et al., 1999; Villa et al., 2000), that are mostly represented by rural workers in the endemic areas (Franco, 1987). Poor hygiene, malnutrition, smoking, and alcohol consumption are considered risk factors for the manifestation of the disease (Silva-Vergara et al., 2000). The inhalation of the fungus conidia or mycelial propagules is the most common transmission mode that allows the fungi to reach the lungs (which are the primary infection site).

The clinical forms of PCM were classified based on the relationship between clinical aspects and the natural history of the disease. Then, the infection is related to the patient without signals and symptoms of the disease but with a positive paracoccidioidin skin test reaction. Patients with PCM disease were divided between acute/subacute form (juvenile type), that mainly affects children and young adults presenting more disseminated lesions, and chronic form (that primarily affect adult men) generally found in oral mucosa, airways, and lung lesions (Bocca et al., 2013; Marques, 2013; Martinez, 2015).

Because of its importance, this review will summarize all the recent discoveries on *Paracoccidioides*-host interaction with particular emphasis in fungi surface proteins, which play a fundamental role in the adhesion and/or dissemination of the fungi to host-cells. The goal is to point out that there are multi-factorial processes involving host–pathogen interactions as well as fungal virulence and host immune response. Finally, this review will also focus in the recent advances in drug discoveries and treatments of PCM.

THE ADHESION PROCESS IN PCM

The interaction of host and parasite is a complex event in which the host is under pressure to develop resistance while the parasite tries to evade and adapt to the host's immune response and thus survive in the host's environment (Tronchin et al., 2008; Sironi et al., 2015).

Paracoccidioidomycosis can be acquired through inhalation of infectious propagules, which then lodge in the alveoli from which they spread to other organs. The fungi developed mechanisms (such as adhesion to host cells), to avoid entrapment within mucus and their elimination by mucigen cilliary cells (Filler and Sheppard, 2006; Hernández et al., 2010). Therefore, their effective adherence contributes to higher speed invasion of host cells, allowing for evasion of the immune system, establishment of the infection, and in the case of *Paracoccidioides* spp., lead to different clinical manifestations (Singer-Vermes et al., 1994; de Oliveira et al., 2015). Another important fact is that *P. brasiliensis* is able to form biofilm *in vitro*, which opens up new possibilities in understanding the infection process of these fungi, since biofilm formation is a condition that provides for the pathogen's protection against drugs and the host's immune system (Sardi et al., 2015).

Some differences in the degree of adherence have been observed for *Paracoccidioides* spp. regarding the manner in which they enter different cell types. This is perhaps related to changes in the cell wall composition (Telles-Filho, 1987; Puccia et al., 2011). Hanna et al. (2000) observed differences in adhesion capacity to Vero cells of four *P. brasiliensis* strains. Additionally, successive subcultures of *P. brasiliensis* resulted in their attenuation or loss of virulence (Brummer et al., 1990), that can be re-established

TABLE 1 | Occurrence of paracoccidioidomycosis in the Brazilian territory raised by eco-epidemiological studies.

State	Region	Period	Number of cases	Gender/age	Reference
Parana	Western	2008–2009	102	72 male and 30 female/18–81 years	Loth et al., 2011
Sao Paulo	Southeast	1960–1999	1.000	858 male and 142 female/03–80 years	Bellissimo-Rodrigues et al., 2011
Amazon	North	1997–2012	2.163	1.951 males and 211 females/03–81 years	Vieira et al., 2014
Sao Paulo	Southeast	1988–1996	584	492 males and 92 females/05–87 years	Blotta et al., 1999
Rio Grande do Sul	South	1966–2009	123	104 males and 17 females/02–97 years	de Souza et al., 2014

after passage in animals (San-Blas et al., 1977; Castaneda et al., 1987) or epithelial culture cells (Andreotti et al., 2005).

Recently de Oliveira et al. (2015) found significantly higher capacity for adhesion to pneumocytes by *P. brasiliensis* compared to *P. lutzii*. They also demonstrated that *P. brasiliensis* is more virulent than *P. lutzii*, using an *in vivo* model. This supports the fact that adherence and virulence are closely related in *Paracoccidioides* spp. and reinforces the importance of adhesion in the infection process of these fungi.

Paracoccidioides spp. recognizes several of the host molecules such as components of the extracellular matrix (ECM). ECM is basically composed by collagen, elastin fibers, glycosaminoglycans (GAGS), proteoglycans (PG), fibronectin, laminin, heparan sulfate, nidogen/entactin, hyaluronate, chondroitin sulfate, and collagens subtypes I, III, IV, and V (Dunsmore and Rannels, 1996; Mendes-Giannini et al., 2006; Balestrini and Niklason, 2015). These can serve as a substrate for the attachment of colonizing microorganisms (Chagnot et al., 2012). Comparing the adhesion to ECM components of two species of *Paracoccidioides*, de Oliveira et al. (2015) demonstrated that *P. brasiliensis* adheres more to fibronectin in contrast to *P. lutzii* that showed more tropism to type I and IV collagen.

Different studies reinforced the importance of the fungus interactions with ECM proteins during the adhesion process. González et al. (2008b) evaluated *Paracoccidioides* conidia treated with laminin, fibronectin, and fibrinogen in mice experimental PCM. They observed that the treatment with all ECM proteins, especially laminin and fibrinogen, induces a less severe pathology, with a decrease of chitin content in the lungs. In a different study, André et al. (2004) treated yeasts of *Paracoccidioides* with laminin and they to observed that this treatment induces a less severe pathology.

Several studies in the *Paracoccidioides* genus have been conducted to characterize the adhesion process, revealing that *Paracoccidioides* spp. synthetizes several molecules, known as surface adhesins, that are involved, directly or indirectly, in the interaction with host cells and in the *in vitro* biofilm formation (Mendes-Giannini et al., 2000; Hernández et al., 2010; Sardi et al., 2015).

Paracoccidioides spp. adhesins are widely studied using *in vitro* and *in vivo* approaches of different forms of *Paracoccidioides* spp. in order to identify and characterize these molecules. The understanding of the adhesion process provides a better understanding of the disease as well-bringing new possible targets for therapeutics (Gonzalez et al., 2005; Tomazett et al., 2005; Pereira et al., 2007; de Oliveira et al., 2015).

Several *Paracocccidiodes* spp. adhesins have been found to have a multifunctional role, being primarly involved in metabolic pathways and later found in fungus cell walls and/or secreted and mediating fungus adhesion (Karkowska-Kuleta and Kozik, 2014). The transport of these molecules (together with antigenic components and other molecules that can interact with the host's cellular immune system such as α-galactosyl), to the fungal cell wall and to the extracellular compartment, can be mediated by vesicles produced by the fungus as described by Vallejo et al. (2011, 2012). Recently, Peres da Silva et al. (2015) described the presence of RNA in extracellular vesicles secreted by *Paracoccidioides* spp., which might interfere in both fungi and host gene expression, modulating the host–pathogen interaction.

The gp43 is a glycoprotein that is the most studied molecule from *Paracoccidioides* spp. Due to its importance in the host–pathogen interactions, including the adhesion process. The gp43 was the first described *P. brasiliensis* adhesin, and it binds to laminin and fibronectin. *In vitro* studies showed that treatment with gp43-purified protein is able to reduce *Paracoccidioides* adhesion, showing that this protein is one of the mediators of fungus adhesion to host epithelial cells and internalization (Vicentini et al., 1994; Hanna et al., 2000). This interaction also occurs with macrophages cells. Silenced strains for gp43 are less adherent or internalized by activated macrophages (Almeida et al., 1998; Torres et al., 2013).

A 32 kDa hydrolase (PbHAD32) was found in *Paracoccidioides* spp. cell wall extracts from mycelial and yeast forms and is able to bind to laminin, fibronectin, and fibrinogen and act as an adhesin in the initial stage of *Paracoccidioides* spp. adhesion. An increase of PbHAD32 is observed during the transition from conidia to yeast or mycelial form (González et al., 2005, 2008a; Hernández et al., 2012). Hernández et al. (2010) using antisense RNA (aRNA) technology performed PbHAD32 silencing and the knockdown of this gene resulted in morphological changes in the yeast form. Furthermore, a decrease of the adherence of both yeast and conidia forms to epithelial lung cells (A549) in a knockdown strain (*PbHAD32 aRNA)* was observed. *In vivo* analysis demonstrated a significant increase in the survival rate of mice challenged with *Paracoccidioides PbHAD32 aRNA* when compared to the wild type *Paracoccidioides* (Hernández et al., 2010, 2012).

The 30 kDa protein was first identified in a *Paracoccidioides* proteomic study before and after mice infection, where a significant increase of its expression was observed after mice infection and was also characterized as a laminin ligand (Andreotti et al., 2005). Later, da Silva et al. (2013) sequenced this protein and identified it as belonging to the 14-3-3 protein

family characterized as small multifunctional proteins present in eukaryotic cells. During infection, Pb14-3-3 accumulates in the *P. brasiliensis* cell walls. The treatment with recombinant protein, promotes the inhibition of *P. brasiliensis* adhesion to epithelial lung cells, demonstrating its importance in this process (da Silva et al., 2013).

In a study conducted by Donofrio et al. (2009) to identify fibronectin-binding adhesins from *P. brasiliensis,* a 54 kDa protein was highly expressed in different strains when cultured in a medium supplemented with blood. The sequencing of this protein identified it as enolase (PbEno), a well-known glycolytic enzyme. Later, it was found that surface expressed PbEno also binds to laminin, type I and IV collagen, plasminogen, and fibrinogen (Nogueira et al., 2010; Marcos et al., 2012). The participation in the adhesion process was evaluated using anti-54 kDa polyclonal antibodies in which the inhibition of *P. brasiliensis* adhesion to epithelial lung cells *in vitro* was demonstrated (Nogueira et al., 2010). In addition, during infection and when cultivated with sheep blood, an increase of PbEno in cell walls was observed, demonstrating its role in fungus–host interaction (Marcos et al., 2012). The ability of enolase to interact with plasminogen has already been related to the pathogen's invasive capacity. It is mediated by lysine-dependent binding, degrading ECM, and promoting invasion (Ghosh and Jacobs-Lorena, 2011). Sequence analysis showed that PbEno has similar internal motif responsible for plasminogen binding in *Paracoccidioides* spp. and the recombinant PbEno binding to plasminogen in the presence of a lysine, suggesting that PbEno also plays a role in the invasion process (Marcos et al., 2012).

Triosephosphate isomerase (TPI) is a glycolytic enzyme described as a protein that is able to react with the sera of PCM patients. The localization of TPI was detected in the cytoplasm and in the cell wall of the yeast phase of *P. brasiliensis* (da Fonseca et al., 2001). Pereira et al. (2007) produced an anti-TPI polyclonal antibody and used it to treat *P. brasiliensis* and observe the influence of this treatment in the interaction of the fungi with epithelial cells. After the treatment, they observed that the antibody inhibited the adhesion of *P. brasiliensis*. These findings showed the involvement of the TPI in the cell adhesion acting as an adhesin.

Malate synthase is an enzyme from the glyoxylate pathway and in *Paracoccidioides* spp. is also required in allantoin degradation pathway (Zambuzzi-Carvalho et al., 2009). However, besides the metabolic role, PbMLS is found in fungus cell walls and is characterized as an adhesin able to bind to fibronectin as well as types I and IV collagen (da Silva Neto et al., 2009). In a study of intermolecular interactions of PbMLS, de Oliveira et al. (2013) found that PbMLS present in cell walls interact with other adhesins such as enolase and TPI and this interaction could enhance the adhesion ability.

GAPDH (glyceraldehyde 3-phosphate dehydrogenase) is a well-known protein from the glycolysis pathway, however, this protein can also act as virulence factor for some pathogens, including fungal pathogens (Karkowska-Kuleta and Kozik, 2014; Marcos et al., 2014). In *P. brasiliensis*, GAPDH expression is increased during the mycelium-yeast transition and was found in cell wall and extracellular vesicles (Barbosa et al., 2004; Longo et al., 2014). *Paracoccidioides* spp. GAPDH is able to bind to host ECM components laminin, fibronectin, and type I collagen. This molecule seems to play a role in the initial steps of infection once *in vitro* assays demonstrated the inhibition of adhesion and infection of *P. lutzii* to pneumocytes after fungus incubation with anti-GAPDH antibody or cell treatment with recombinant GAPDH (Barbosa et al., 2006).

Another protein involved in the process of cell adhesion and tissue invasion/dissemination is the fructose 1,6 bisphosphate aldolase (ALD) protein that interacts with plasminogen. The antibodies anti-ALD and the recombinant protein were able to reduce the interaction between macrophages and *Paracoccidioides* (Chaves et al., 2015).

Recently, de Oliveira et al. (2015) evaluated gene expression of differents adhesins as 14-3-3, ENO, gp43, MLS, GAPDH, and TPI after mice infection by *P. brasiliensis* and *P. lutzii*. They could observed that 14-3-3 and enolase were the most expressed adhesins and also that *P. brasiliensis* express in higher levels adhesins than *P. lutzii*. Besides this, this study demonstrated that the virulence of its species is related to its adhesion capacity with *P. brasiliensis* being more virulent than *P. lutzii*.

The adhesion is an universal prerequisite for pathogens to efficiently deploy their repertoire of virulence (Krachler and Orth, 2013). In summary, the attachment of *Paracoccidioides* spp. to host cells is mediated by adhesins present at the fungal surface and that this is a critical step in PCM, acting as an essential virulence factor for *Paracoccidioides* spp. **Figure 1** summarizes the affinity of each adhesin to different ECM components: laminin, fibronectin, type I collagen, type IV collagen, plasminogen, and fibrinogen.

Based on all that was presented in this section, what is needed are new approaches that aim for the discovery of new molecules or further investigation of the already known molecules. This holds especially true for studies that evaluate strategies to block the adhesion in order to try and discover how the fungi modulated itself to cause the infection and how we can avoid the infection and prevent the PCM.

MORPHOLOGICAL SWITCHING AND PATHOGENICITY OF *Paracoccidioides* spp.

Different polysaccharides, proteins, lipids, and melanin compose the complicated structure of fungi cell walls. An incisive way for pathogens to respond, adapt, and survive in new niches of infection can be found in the alterations in the expression of surface-exposed molecules under different environmental conditions (Karkowska-Kuleta and Kozik, 2015).

The genus *Paracoccidioides* comprises of thermally dimorphic fungi that grow as saprophytic mycelium at environmental temperature (e.g., 26°C; San-Blas, 1993). Produces infective conidia or mycelial (M) fragment propagules that are inhaled by the host. When the propagules reach the pulmonary alveolar epithelium of a mammalian host (exposed to temperature higher than 37°C), they transform into the parasitic yeast (Y) form

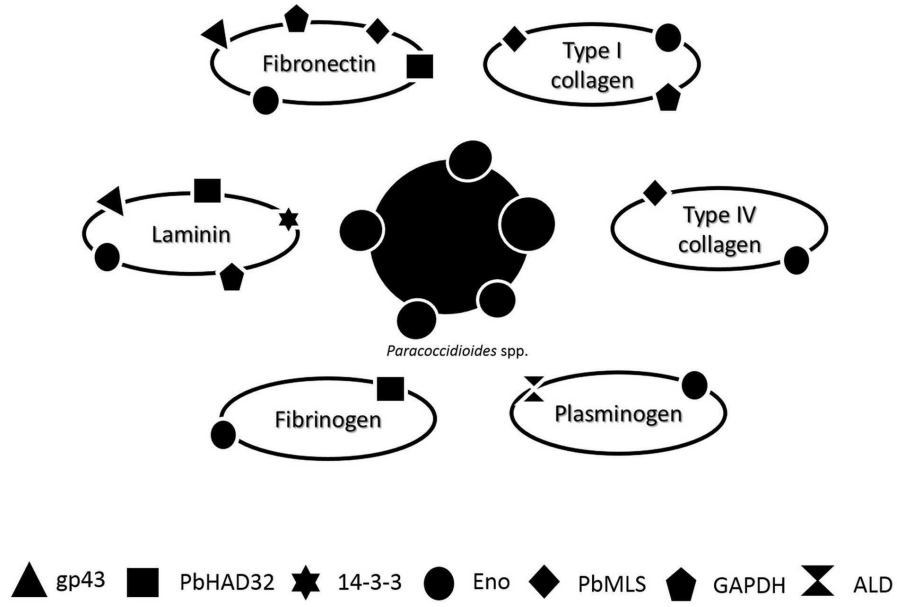

FIGURE 1 | Schematic representation of the affinity of different *Paracoccidioides* spp. adhesin [gp43, hydrolase (PbHAD32), 14-3-3, Enolase (ENO), Malate synthase (MLT), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 1,6 bisphosphate aldolase (ALD)] to different ECM components: laminin, fibronectin, type I collagen, type IV collagen, plasminogen, and fibrinogen.

causing different clinical manifestations. This phenomenon can also be reversibly triggered *in vitro* by changing the temperature from 26 to 37°C (San-Blas et al., 2002).

The glycoproteins and polysaccharides network that composes the cell wall of *Paracoccidioides* spp., similar to many other fungi, is important for the protection against environmental stresses (De Groot et al., 2005) while promoting its virulence. The morphological switch, a mechanism for allowing the pathogen to adapt and thrive inside the host (Nemecek et al., 2006), is obligatory for the establishment of the infection. This seems to be related to pathogenicity, since isolates incapable of undergoing the morphological transition are less virulent (Maresca and Kobayashi, 2000).

The fungal cell wall synthesis is performed by glucan synthases (Sorais et al., 2010). The phenotypic switch entails changes in the composition of the fungal cell walls with a predominance of β-1,3 and β-1,6-glucan and carbohydrates in mycelial form, while in yeast form there is a prevalence of α-1,3-glucan and chitin (Puccia et al., 2011; Free, 2013). Changes may occur in the quantity and the spatial arrangement of these polysaccharides (San-Blas et al., 1994). This occurs in order to ensure fungal survival within the host (Tavares et al., 2015). α-1,3-glucan is correlated with the level of virulence (Hogan and Klein, 1994), hiding immunostimulatory β-glucans that could be detected by the host phagocytic cells (Klein and Tebbets, 2007), assisting its evasion of the host's immune responses (Rappleye et al., 2007).

For *Paracoccidioides* spp. the temperature is an essential factor involved in dimorphism and is preceded by several molecular changes (Boyce and Andrianopoulos, 2015). This is a characteristic shared with other dimorphic fungi such as *Histoplasma capsulatum*, *Blastomyces dermatitidis* (Medoff et al., 1987), *Coccidioides immitis, Sporothrix schenckii,* and *Penicillium marneffei* (Klein and Tebbets, 2007). The growth of yeast from the mycelium is initiated from the time of thermal change reaching 50% of changes after 48 h from the start of the process. The multiple developments into yeast budding occurs after 5 days (Salazar and Restrepo, 1985; Goldman et al., 2003). The reverse has also been reported and studies show that 48 h following the temperature change, 50–60% of the cells showed conversion mycelium (Goldman et al., 2003; Nunes et al., 2005).

However, in *Paracoccidioides* genus the conidia- or mycelium-to-yeast transition is blocked by exposure of *Paracoccidioides* spp. to female hormones, such as estrogen, via steroid-binding proteins (Loose et al., 1983; Aristizábal et al., 1998). This gives the PCM the peculiarity of affecting more men compared to the number of women (Shankar et al., 2011b).

Regarding the evaluation of molecular mechanisms and gene expression in both morphological states, several studies have been conducted using different strategies such as expressed sequence tags (ESTs) libraries, microarrays, analysis of genes expressed during the stages of mycelium and yeast, as well as those differentially expressed in transition and proteomics. Several efforts have been made to understand the morphological alterations, including those depending on the factors of temperature and the presence of female hormones. There are many studies focusing on genes that are regulated during mycelium-to-yeast (M-Y) transition (Felipe et al., 2005; Bastos et al., 2007; Parente et al., 2008; Muñoz et al., 2014). Although a number of genes that govern the phase transition are known, how these genes fit into a larger network of regulated genes remains poorly explored (Gauthier, 2015).

Transcriptional analysis of 6,022 assembled groups demonstrated that the mycelial cells have a more aerobic metabolism in comparison to the yeast phase, with greater expression of genes of citrate cycle such as succinyl-CoA dehydrogenase and isocitrate synthase suggesting a metabolic shift to oxidative phosphorylation. In contrast the yeast phase displays slanted energy metabolism for the production of alcohol by fermentation, presenting the glyoxylate pathway (into anaerobic metabolism) as being more active. This is demonstrated by analysis of transcription in yeast and mycelia which is consistent with low oxygen levels found in infected tissue (Felipe et al., 2005).

A biochip carrying of 4,692 genes from *P. brasiliensis* was used to trace gene expression in different points of the morphological transition (5–120 h). Among the various genes identified, some encoding enzymes are involved in the catabolism of amino acids, signal transduction, protein synthesis, cell wall metabolism, genome structure, response to oxidative stress, growth control and development (Nunes et al., 2005).

Proteomic analysis during phase conversion of *P. brasiliensis* demonstrated quantitative differences correlated with transcripts levels. The mycelia phase protein profile showed 18 overexpressed proteins involved in cell defense, energy, and protein fate. During M-Y transition, 33 proteins were upregulated, most of them belonging to the glycolytic pathway. Some glycolytic enzymes such as enolase and fosfoglucomutase begin to accumulate during the transition (M-L) and maintain high levels in the yeast phase. It is therefore another sign of the global reorganization of carbohydrate metabolism that occurs during morphological change (Rezende et al., 2011).

N-*linker* glycans are involved in glycoprotein folding, intracellular transport, secretion, and protection from proteolytic degradation (Nagai et al., 1997). In *Candida albicans* it has been shown to be involved in cell wall integrity as well as in the fungus–host interaction (Mora-Montes et al., 2007). Dos Reis Almeida et al. (2014) showed that the treatment of *Paracoccidioides* with tunicamycin, responsible for blocking the N-*linked* glycosylation of α-1,4 amylase, interfere in the transition for both Y-M and M-Y, since the α-1,4 amylase is responsible for biosynthesis of α-1,3 glucan the major cell wall glucan of the yeast form.

Phosphatidic acid and diacylglycerol produced by a phospholipase D1 participates in the morphological transition of *C. albicans* (Hube et al., 2001). A similar finding was seen in *P. brasiliensis* in which up-regulation of phospholipase was found in M-Y transition (Soares et al., 2013).

Thermal dimorphism may occur as a result of a specialized heat shock response triggering a cellular adaptation to high temperatures (Lambowitz et al., 1983). Matos et al. (2013) demonstrated the involvement of HSP90 during the dimorphism of *P. brasiliensis* using pharmacological approaches. HSP90 is required for the transition from non-infective to infective forms but not for Y-M transition. This protein is also highly transcribed under *in vitro* oxidative stress. HSP90 is a chaperone that binds and stabilizes calcineurin. It also competes with calmodulin for the Ca^{2+}/calmodulin docking site in calcineurin interfering with the activation of the latter (Imai and Yahara, 2000). So, it was suggested that HSP90 acts synergistically with calcineurin in the control of cell differentiation (Matos et al., 2013). Other proteins of HSP family such as HSP70, HSP80, and HSP88, were down-regulated in mycelial cells treated with estradiol (Shankar et al., 2011b) suggesting that this hormone impairs the favorable expression of genes necessary for adaptation to a change of temperature (Nicola et al., 2008).

In spite of several studies suggesting the potential role of estradiol in dimorphism of *Paracoccidioides*, the exact mechanism that leads to such genetic modulation resulting in differences in disease rates, remains unknown (Tavares et al., 2015). Hormones act as messenger molecules, leading to regulation of gene expression through receptor-mediated interactions that mediate this interaction and the subsequent functional response to the presence of the hormone (Shankar et al., 2011a).

In vivo studies have shown that female mice, especially at estrus, reach a higher clearance of yeast and restraint of fungal proliferation as compared to male mice (Aristizábal et al., 1998, 2002; Sano et al., 1999). Pinzan et al. (2010) revealed remarkable influences of gender on experimental PCM, which could be partly attributed to interference of female hormones on the immune response triggered by a *P. brasiliensis* infection. Estradiol promotes protective responses to this infection, IL-12, IFN-γ, and TNF-α cytokines (Calich et al., 1998; Kashino et al., 2000) correlated with resistance to female infection. On the contrary, infected male produces IL-10 (Benard et al., 2001; Oliveira et al., 2002; Romano et al., 2002) which plays an important role in antigen specific immunosuppression of PCM (Pinzan et al., 2010).

Shankar et al. (2011b) using microarray technology to evaluate the *P. lutzii* transcriptional response to a fixed concentration of estradiol during 9 days, revealed that the chitin synthase 1 gene (CHS) was down-regulated in response to estradiol at earlier time points. Nunes et al. (2005) identified a positive modulation of chitin synthases and down-regulation of chitinases in the M–Y transition, while Bastos et al. (2007) detected two chitinases over-expressed in the dimorphic transition. In fact, the yeast cell wall is mainly constituted of chitin (37–48%), compared to the mycelium form (7–18%; Kanetsuna et al., 1969).

High levels of gene expression may occur during this process. Hernández et al. (2011b) showed increased expression of HSP90, AOX, and GS1 (glucan synthase-1) throughout the entire yeast to mycelium germination and αGS (glucan synthase α) for the opposite. The HSP90 was up regulated early in the transition suggesting their involvement in the initial contact of the fungus with the host and the modifications necessary to adapt within the same. The AOX (alternative ubiquinol oxidase) gene acts by reducing the reactive oxygen species and correlates with metabolic activation required to obtain carbon and energy owing to the non-phosphorylative nature of the alternative respiratory pathway to the morphological changes (Gessler et al., 2007; Hernández et al., 2011a). AOX is present in early stages of M-Y transition and plays an important role in intracellular redox balance. Furthermore, it is the only enzyme in *P. brasiliensis* that is not present in its mammalian hosts therefore it is a promising target for therapy (Martins et al., 2011).

TNF-α is a cytokine related with anti-microbicidal activity in IFN-γ activated macrophages stimulating NO production. Gonzalez et al. (2004) illustrated that TNF-α activated macrophages are capable of inhibiting the conidia to-yeast transition in *P. brasiliensis* by an NO-independent pathway; acting as a fungicidal and/or fungistatic mechanism against *P. brasiliensis* conidia.

Cyclic AMP (cAMP) is the regulatory component of a well-characterized signaling pathway implicated in a variety of cellular processes among fungal species (Fernandes et al., 2005). The importance of the cAMP-signaling pathway in the control of morphological changes and pathogenicity of various fungi has been reported (Medoff et al., 1987; Cho et al., 1992; Borges-Walmsley and Walmsley, 2000). During morphological transition the cAMP levels increase transiently in the early stages (<24 h) and progressively in the subsequent stages (>120 h; Chen et al., 2007). Moreover, the transition may be modulated by exogenous cAMP (Paris and Duran, 1985; Chen et al., 2007), suggesting possible involvement of cAMP in the dimorphic transition.

Understanding how different cell types recognize both yeast and mycelial and how each cell type is activated in accordance with the morphology is important, however, the likely consequences of this activation probably differ according to cell type (e.g., in macrophage or in epithelial cell; Jacobsen et al., 2012). Inhibitors targeting the morphological transition from mycelium-to- yeast are an interesting choice to attempt to control the *Paracoccidioides* infection. As discussed above the change to yeast form is essential for the establishment of infection, and thereby inhibitors of this can prevent the infection. **Table 2** summarizes the main works related to dimorphism of *Paracoccidioides* spp. that focused on specific genes and proteins.

HOST CELLS MANIPULATION BY *Paracoccidioides* spp.

The ability of pathogens to colonize their hosts is highly dependent on mechanisms that may allow the pathogen to break the physical and immunological barriers imposed by the host. In order to avoid rapid clearance of the organism, pathogens act quickly and effectively on adhesion to host cells.

The capacity of cells to interact with each other in an orderly manner depends on multiple adhesive interactions between cells and their adjacent extracellular environment, mediated by cell adhesion molecules (Miyoshi and Takai, 2008; Troyanovsky, 2009). These function as cell surface receptors that can trigger physical and biochemical signals that regulate a great numbers of functions such as cell proliferation, gene expression, differentiation, apoptosis, and cell migration and are used as a gateway to some pathogens (Finlay and Falkow, 1997; Guttman and Finlay, 2009; Han et al., 2010; Voltan et al., 2013).

Many pathogenic microorganisms have the ability to induce its internalization in epithelial cells, forcing the activation of phagocytosis mechanism. Specific extracellular signals can stimulate their cytoskeleton rearrangement in the contact site with the microorganism, making cells to behave like a "phagocyte unprofessional" (Swanson and Baer, 1995; Lim and Gleeson, 2011), in a process that involves integrins and the cytoskeleton (Mendes-Giannini et al., 2004; Feriotti et al., 2013). *Paracoccidioides* spp. invasion affects the structure of the cytoskeleton of pulmonary epithelial cells and keratinocytes, interfering with morphological aspects of actin, tubulin and cytokeratin (Mendes-Giannini et al., 2004; Peres da Silva et al., 2011).

The capacity of fungal invasion to mammalian cells is specific to some fungi and there is still a lacuna in the understanding of this process (Tsarfaty et al., 2000; Wasylnka and Moore, 2002; Mendes-Giannini et al., 2004). The signaling pathways that control the morphological changes in *P. brasiliensis*, as well as the cellular signals upon interaction with the host cell are also not well-understood. Mendes-Giannini et al. (2004) showed that treatment with cytochalasin D and colchicine reduced the invasion by *P. brasiliensis*, indicating the functional involvement of microfilaments and microtubules in this process.

Some studies evaluated the role of adhesins in the invasion process of *Paracoccidioides* and it was observed that gp43 may also participate in the cytokeratin degradation leading to the loss of the filamentous characteristics that can facilitate the invasion of the host (Mendes-Giannini et al., 1990; Puccia and Travassos, 1991; Tacco et al., 2009; Puccia et al., 2011). The 14-3-3 adhesin is also known to have the capacity to cause structural modifications in the host cells influencing in the polymerization of the cytokeratin microfilaments of actin (Andreotti et al., 2005; Mendes-Giannini et al., 2006; da Silva et al., 2013).

The Rho GTPase family of proteins is known to regulate the dynamic organization of the cytoskeleton and membrane traffic physiological processes such as cell proliferation, motility, polarity, and growth (Sinha and Yang, 2008). The Rho-GTPase is able to down-regulate genes related to chitin and glucans biosynthesis. Rho-GTPase, FKS (β-1,3 glucan synthase), and AGS1 (coding α-1,3 glucan synthase) were down-regulated on *Paracoccidioides* estradiol samples treated. They indicate that this hormone promotes a transcriptional modulation of the cell wall, remodeling related genes (Shankar et al., 2011b; Tavares et al., 2015). Rho GTPases have been extensively studied in human fungal pathogens and have a set of interacting proteins to orchestrate their activation in the cells (Yamochi et al., 1994). Cdc42, a member of the Rho GTPase, was characterized as a convergence point in the signal transduction and are involved in multiple signaling pathways including receptor tyrosine kinases and cytokines, heterodimeric G proteins, as well as physical and chemical stress. In *P. brasiliensis* plays a role in morphology of yeasts cells since the knockdown PbCDC42 showed decrease in cell size and more homogenous cell growth and this provided a higher phagocytosis and decreased virulence (Almeida et al., 2009).

In mammalian cells, the Rho GTPases are also the center of a complex signaling pathway that plays an important role in adhesion. The activation of tyrosine kinase (PTK) receptors stimulate Rho GTPase which in turn activates the Ras pathways and MAPKs (Sinha and Yang, 2008). Monteiro da Silva et al. (2007), showed significant inhibition of fungal invasion after pre-treatment of epithelial cells with genistein, a specific inhibitor

TABLE 2 | Summary of studies related to dimorphism of *Paracoccidioides* genus.

Condition	Approach (isolate)	Target	Observation	Reference
Mycelium-to-yeast transition	Pharmacologycal tools – inhibition with geldanamycin (*P. brasiliensis)*	Hsp90	Up-regulated	Matos et al., 2013
Mycelium-to-yeast Yeast-to-mycleium	Real-time reverse transcription-polymerase chain reaction	Hsp90/AOX/GS1 αGS	Up-regulated Up-regulated	Hernández et al., 2011b
Mycelium-to-yeast transition	Pharmacologycal tools – inhibition with CsA, a calcineurin inhibitor cyclosporine A (*P. brasiliensis)*	Calcineurin	Transition to the yeast form was blocked before the blastoconidial budding stage	Campos et al., 2008
Mycelium-to-yeast transition	Transcriptional profiling and pharmacological tools – 4-HPPD inhibitor (*P. brasiliensis*)	4-hydroxyl-phenyl pyruvate dioxygenase (4-HPPD)	Up-regulated Inhibit growth and differentiation to the pathogenic yeast phase	Nunes et al., 2005
Mycelium-to-yeast	Pharmacologycal tools – inhibition with benzohydroxamic acid – inhibitor of AOX (*P. brasiliensis)*	AOX	Delayed the M-Y transition	Martins et al., 2011
Yeast-mycelium germination and mycelium/conidia-to-yeast transition	Antisense RNA technology (*P. brasiliensis*)	AOX	Delayed the Y-M transition	Hernández et al., 2015
Mycelium-to-yeast transition	Transcriptional response to 17-β-estradiol treatment (*P. brasiliensis)*	Chitin synthase	Down-regulated in response to estradiol	Nunes et al., 2005
Mycelium-to-yeast transition	1007 ESTs from a transition cDNA library (*P. lutzii*)	Two chitinases	Up-expressed	Bastos et al., 2007
Mycelium-to-yeast transition	Gene expression in the presence or absence of 17-β-estradiol (*P. lutzii*)	Rho-GTPase components FKS1 and AGS (coding α-1,3-glucan synthase)	Down-regulated	Shankar et al., 2011b
Mycelium-to-yeast and yeast-to-mycelia	Pharmacological approach – using tunicamycin (TM)	N-glycosylation	TM treatment interferes the transition in both directions by interference in the activity of α-1,4 amylase (involved in the biosynthesis of α-1,3 glucan)	Dos Reis Almeida et al., 2014
Mycelium-to-yeast	Real-time (*P. brasiliensis*)	Phospholipase (PLB)	Up-regulation in mycelial cells	Soares et al., 2013

of PTK located on the plasma membrane of the epithelial cells. These results suggest that the inhibition of PTK is important in signal transduction during early events in the adhesion and invasion processes of *P. brasiliensis* in epithelial cells.

Apoptosis is a highly regulated physiological process of cell death required for the development and homeostasis of multicellular organisms by eliminating individual cells without inducing an inflammatory response. The enabling or prevention of apoptosis can be a critical step in the development of infectious processes. The process of apoptosis is characterized by typical changes in the symmetry of the plasma membrane, chromatin condensation, nuclear fragmentation, DNA cleavage, cell disintegration, and formation of apoptotic bodies (Strasser et al., 2011).

Programmed cell death has been observed as a response to a variety of infections and can be mediated by a variety of virulence determinants encoded by pathogens. The modulation induced by pathogens pathways responsible for cell death in the host favor the elimination of cells of the immune system or avoidance of host defense response that attempt to act in eliminating the infection (Weinrauch and Zychlinsky, 1999).

The ability of the pathogen to induce apoptosis in phagocytes may be an important virulence factor, since it reduces the host's defense mechanisms (Ashida et al., 2011). *P. brasiliensis* and other fungi can induce the apoptosis of phagocytes to acquire advantages, allowing intracellular survival in epithelial cells (Cacere et al., 2002).

Paracoccidiodes brasiliensis induces apoptosis when it invades epithelial cells or phagocytes, which benefits its intracellular survival (Souto et al., 2003; Mendes-Giannini et al., 2004, 2005; Verícimo et al., 2006; Ketelut-Carneiro et al., 2015). Silva et al. (2008), showed that *P. brasiliensis* induces apoptosis of macrophages by expression of caspase-2, 3 and 8, but also found that it induces the expression of genes that encode inhibitors of apoptosis proteins, such as caspase-8 and Fas-L inhibitors. Caspases-2 and 8 are responsible for the transduction of signals for cleavage of other caspases, such as caspase-3, which leads to apoptosis induction (Silva et al., 2008).

Paracoccidiodes brasiliensis may modulate apoptosis of epithelial cells A549 by the expression of apoptotic molecules such as Bcl-2, Bak, and caspase-3, confirming the inducing of apoptosis by the fungus which can then survive and spread to other parts of the body (Del Vecchio et al., 2009). More recently, Silva et al. (2015), showed that the 14-3-3 and gp43 adhesins has strong influence in this process. Campanelli et al. (2003) demonstrated that apoptosis mediated by Fas-FasL and CTLA-4 engagement are involved in modulating the immune response in patients infected with PCM. Souto et al. (2003) demonstrated in experimental PCM, a considerable increase in apoptosis in the infection site. Cacere et al. (2002) studied the role of apoptosis

in PCM using peripheral blood mononuclear cells of patients with the PCM disease, noting that apoptosis induced by gp43 was lower in controls than in peripheral blood mononuclear cells of patients.

All of these studies demonstrated that *Paracoccidioides* spp., during its evolution, has developed mechanisms that allows the fungi, since it is inhaled in its mycelial form, to survive in a hostile host environment. During the interaction, using its adhesins, *Paracoccidioides* spp. adheres to host ECM components and cells, manipulates the cell cytoskeleton, invades the cytoplasmic compartment, and can then induce the cell apoptosis, which gives it its capacity to evade the immune system and spread within the host organism causing systemic mycosis, as summarized in **Figure 2**.

ADVANCES IN ANIMAL EXPERIMENTATION FOR THE STUDY OF THE PCM

The use of animals in research is essential for studies of host–fungal interaction, pathogenesis, treatment mechanisms, immunological aspects, or studies that imply the response of a complex organism. It is important to consider the experimental animal type, age, sex, and routes of inoculation (Conti-Diaz et al., 1959; Iabuki and Montenegro, 1979; Defaveri et al., 1982). The firsts studies described guinea-pigs (de Brito and Netto, 1963), hamsters (Iabuki and Montenegro, 1979), and rabbits (Conti-Diaz et al., 1959) as models to study PCM, however, most of the animals do not develop the disease until a long time after being infected (Linares and Friedman, 1972).

After many decades of investigation, murine animal models are considered the gold standard for *in vivo* studies to simulate the *Paracoccidioides* spp. infection. The establishment of a pulmonary PCM was described in murine models using the intratracheal route after about 30 days of infection. Antibodies were detected 15–60 days after infection, however, were not observed after 360 days (Defaveri et al., 1982). Intranasal route was efficient to develop fatal acute pulmonary or chronic pulmonary and disseminated PCM by using different inoculum concentrations. The development of this model of infection is useful to study treatment (fatal acute pulmonary) and to understand immunological aspects of the disease (chronic; Brummer et al., 1984). The dissemination occurs through a hematogenous route and affects mainly the lungs, secondly liver, thirdly the lymph-nodes and finally within the spleen with the formation of granuloma (Bedoya et al., 1986). The classification of nine different congenital strains of mice were realized after infecting these animals using an intraperitoneally route with *P. brasiliensis*. These mice were classified according to the susceptibility to infection as very resistant, intermediate, and sensitive. The study demonstrated that the susceptibility to *P. brasiliensis* infection of the different animal strains was not dependent on the inoculum concentration. In addition, male mice were generally more susceptible to the infection than females (Calich et al., 1985). In the infection of susceptible mice, high numbers of viable yeasts in different organs were found, however, low fungal burden were observed in all examined organs of resistant animals representing the regression of the infection (Singer-Vermes et al., 1993).

Once the infection model was established to study the efficacy of traditional medicines, new drug candidates or drug combinations could be evaluated (Lefler et al., 1985; McEwen et al., 1985; Restrepo et al., 1992), as well as to study immunologic aspects (Defaveri et al., 1989; Calich and Kashino, 1998; Kashino et al., 2000; Pinto et al., 2006).

Because of the advances in medical technology the number of animals in the research increased and many acts and laws were created in different countries to control ethical issues and to minimize the pain to animals during experimentation (Doke and Dhawale, 2015). Since 1959, the use of animals during scientific experiments has been a debated from which the three Rs theory was created. This theory proposes for the **R**eduction of the number of animals used in an experiment, the **R**efinement of the experiment to animal welfare, and the **R**eplacement of animals by using alternative methodologies (Russell and Burch, 1959).

The use of alternative models like tissues or cell cultures, computer or mathematical analysis (*in silico* testing), and imaging/analyzing techniques are suggested to obtain preliminary data before the start of *in vivo* assays. However, in many cases the research requires information about the response of one whole and complex organism (Balls, 2002; Arora et al., 2011; Doke and Dhawale, 2015).

Considering the need for animal experimentation and the restriction in the use of mammalian animals because of ethical issues, researchers developed alternative animal models. Invertebrates represent a good alternative for *in vivo* assays, because of their short life cycle, small size, evolutionary conservation of the innate immune response between invertebrates and mammals, and low cost. A large number of animals can be used per experiment and until now, no ethical problems limit their use (Mylonakis et al., 2007; Lionakis, 2011; Wilson-Sanders, 2011; Glavis-Bloom et al., 2012; Arvanitis et al., 2013).

Different invertebrate model have been used to study fungal diseases. The fly, *Drosophila melanogaster* (Alarco et al., 2004; Lionakis and Kontoyiannis, 2012), the nematode, *Caenorhabditis elegans* (Pukkila-Worley et al., 2009, 2011; Muhammed et al., 2012); the insect *Galleria mellonella* (Fuchs et al., 2010; Mesa-Arango et al., 2013; Firacative et al., 2014; Maurer et al., 2015) were reported as being used to study fungal virulence factors and to identify novel antifungal compounds.

The use of alternative animal models to study PCM was firstly described by Thomaz et al. (2013). In this study, *G. mellonella* model was used to study *P. lutzii* that was able to kill larvae at 25 and 37°C. Moreover, melanization and granuloma-like structures were observed. Recently, because of the new classification of two distinct species (*P. brasiliensis* and *P. lutzii*), a comparative study of the virulence was developed. Both species cause a hemocyte decrease and kill *G. mellonella* in a similar way. However, *P. lutzii* has higher adhesion ability to hemocytes and this could be attributed to the higher expression of the gp43 gene (Scorzoni et al., 2015). To study the importance of the adhesins for the virulence of *P. brasiliensis* and *P. lutzii*, the treatment of

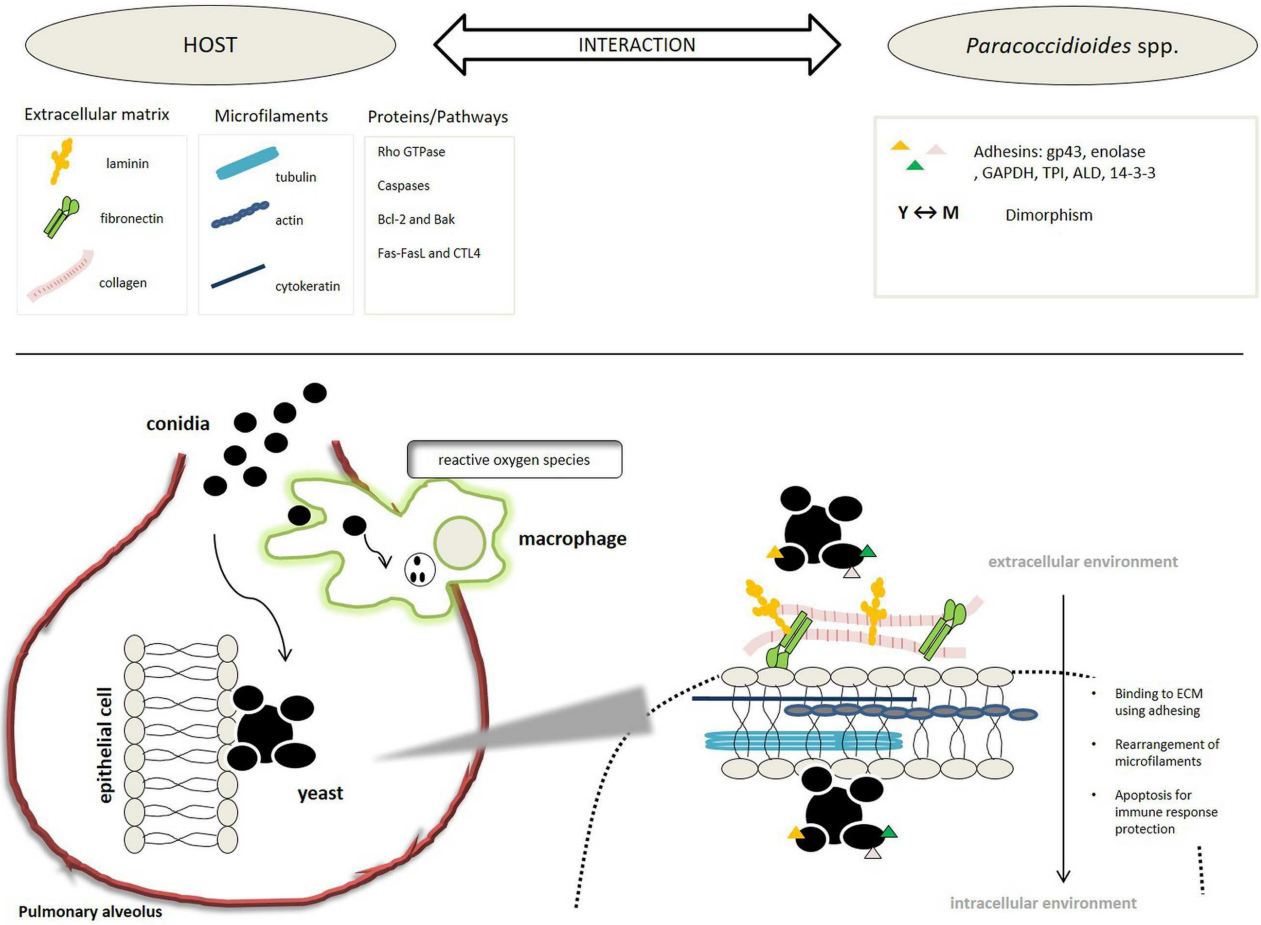

FIGURE 2 | Schematic representation of the steps, processes, and molecules involved in the *Paracoccidioides*-host interaction.

Paracoccidioides spp. with antibodies to block two adhesins (14-3-3 and enolase) caused a decrease in the death of the larvae (de Oliveira et al., 2015).

Despite the evolutionary distance between invertebrate and mammalian models, recent works describe the correlation response to infection between these models (Brennan et al., 2002; Brunke et al., 2015; Desalermos et al., 2015). Considering these evidences, invertebrate models are a good alternative for preliminary studies to investigate *Paracoccidioides* spp. virulence, as well as new treatment and immunological aspects of the infection.

FACING THE PROBLEM: THROUGH THE DIAGNOSTIC TO TREATMENT OF PCM

The diagnosis of PCM is based on clinical and laboratory findings. In the acute or juvenile form of the disease, the skin lesions are often present. On the other hand, in the chronic or adult form, the lung is mainly affected. In this case, it is indicated by a radiography of the organ, which exhibits a pattern that resembles a butterfly wing, characterized by bilateral and symmetrical reticulonodular infiltrate in the two upper thirds of the lungs (Barreto et al., 2012). In the laboratory, the direct microscopic examination of the material collected from lesions or tissue is made to observe the fungi, especially its typical multiple budding aspect, as well as the culture to observe the thermal dimorphism, but the fungus has slow growth (Mendes-Giannini and Fusco-Almeida, 2013; Benard and Mendes-Giannini, 2014).

Furthermore, the diagnosis of disease can be made using serological methods. The counter-immunoelectrophoresis (CID) and double immunodiffusion (IDD) are the reactions most used in reference centers (Vidal et al., 2014). In these cases, the 43 kDa glycoprotein (gp43), which is secreted during the infection, is the main antigen detected. The values of titers correlate with the severity of the disease and efficacy of the treatments. In addition, the negativity or stabilization at dilution 1:2 or less indicates the disease cure (Abreu e Silva et al., 2013). However, differences in the antigenic composition, probably related to phylogenetic peculiarities of the two species, should be considered in the diagnosis of PCM (Batista et al., 2010).

When detection by microscopy and serology fail, an alternative can be the use of molecular techniques as polymerase

chain reaction (PCR) can be used (due to its greater sensitivity). Several studies have been designed with specific primers to target the genes of the *Paracoccidioides* species. For example, primers for the gp43 antigen were developed to identify *P. brasiliensis* DNA (Gomes et al., 2000). A set of primers for PbITS1s and PbITS3a genes was also used for the detection of the fungus by PCR (Buitrago et al., 2009). Another study reported the use of the primer OPG18, which generates two specific DNA fragments (0.72 and 0.83 kb) for *P. brasiliensis* (San-Blas et al., 2005). Finally, Motoyama et al. (2000) showed that the use of fungal universal primers to target 5.8S and 28S rDNA genes followed by more specific primers (OL3 and UNI-R) for PCR resulted in good identification of *Paracoccidioides* spp.

Recently, Nobrega de Almeida et al. (2015) proposed the use of MALDI TOF MS for *Paracoccidioides* spp. identification. In this study, they analyzed 22 strains, belonging to the two species of the genus. All of the strains were correctly identified. MALDI TOF MS is an interesting tool because of its possibility to adapt to routine laboratories and because the results achieved by this study brings benefits in the clinical and laboratorial studies allowing for the identifying of differences between the diseases caused by this genus.

Besides all well-established methodologies to diagnose the PCM, the diagnostic is not an easy subject. The observation of the fungi in clinical specimens, and growth and reversion to mycelium phase, is difficult in clinical labs. Because of this, until recent times, the serological diagnosis was the most commonly used, since molecular approaches are expensive in countries that PCM occur. However, several recent studies in the characterization of different isolates of *Paracoccidioides* spp. bring difficulties to the serological diagnosis.

Gegembauer et al. (2014) for example, demonstrated that serum from PCM patients infected with *P. brasiliensis* is not able to recognize any antigen from the cell-free preparations of *P. lutzii*, however, serum from patients infected with *P. lutzii* is able to recognize both antigens from *P. lutzii* and *P. brasiliensis*. This means that *P. lutzii* serum is more complex antigenically presenting species-specific antigens and common antigens shared with *P. brasiliensis*. Queiroz Júnior et al. (2014) analyzed the protein/glycoprotein profiles of exoantigens from two clinical isolates of *P. brasiliensis* and three of *P. lutzii* with differences between the species observed. *P. lutzii* exoantigens were different from each other showing high species-specific antigens variability, while *P. brasiliensis* isolates exoantigens present similar protein profiles.

Because of these difficulties in the identification and diagnostic of the PCM with incidence of false negative results (da Silva et al., 2015), this is a public health problem as the number of notifications of the disease can be higher than the numbers we currently have today. In addition, the correct identification of the infection can lead to an efficient treatment. Because of this, new efforts in the identification of serological markers is extremely necessary and one of the great challenge in the study of PCM.

Many drugs are useful in treating PCM. Ribeiro in 1940 suggested the initial treatment with sulfapyridine. Later, Lacaz, and Sampaio proposed the use of amphotericin B in 1958. Barbosa e Vasconcelos, in 1973, recommended the use of a combination of trimethoprim-sulfamethoxazole. Around 1979, Negroni suggested the use of ketoconazole. Restrepo, in 1987 suggested itraconazole and more recently, in 2007, the use of voriconazole was suggested by Queiroz-Telles (Cavalcante et al., 2014).

The treatment depends on the severity of the disease, type of antifungal agent, and the time of use. Despite the limited information on studies with different therapies, the itraconazole therapy is the first choice to control the mild to moderate clinical forms. Since 1987, many groups developed studies with azoles antifungals, which showed a reduction in the symptoms, and that they arrested the progression of the PCM (Negroni et al., 1987a,b,c; Restrepo et al., 1987). However, itraconazole therapy is not easily available in most of the endemic regions. Consequently, the therapy consisting of a trimethoprim-sulfamethoxazole combination (daily for 12 months for mild cases and for 24 months in moderate clinical infections) is a useful option. On the other hand, for severe cases, amphotericin B therapy is the best choice. In case of PCM of the central nervous system, the treatment should be with fluconazole or voriconazole therapy daily for 3–6 months, with a maintenance dose daily for 6–12 months. This is because both have a good penetration through the blood brain barrier (Marques, 2012). Today, ketoconazole is little used for the treatment of this infection because of its severe side effects (hepatotoxicity, loss of libido, inhibition of cortisol production etc; Ferreira, 2009).

The possibility of triazole derivatives interacting with several drugs has to be kept in mind such as antihistamines, antacids, H2 receptor blockers, barbiturates, cyclosporine, diphenylhydantoin, digoxin, cisapride, and rifampicin, among others, as well as the well-known side effects and toxicity (nephrotoxicity, myocardial toxicity, myelotoxicity, etc.) related to amphotericin B which will sometimes require discontinuation of therapy (Ferreira, 2009). In the last 30 years there have been efforts at improving AmB preparation, however, the high costs, neglected clinical data, and alternative antifungal therapies have led to the use of this therapy as a second-line therapy (Laniado-Laborín and Cabrales-Vargas, 2009).

More recently, Rodríguez-Brito et al. (2010) evaluated the susceptibility of *P. brasiliensis* (both at their mycelial and yeast phase), to caspofungin, an antifungal drug of the echinocandin class. For the yeast phase, they found that caspofungin was able to inhibit the growth in 20–65%, while in the mycelial, 75–82%. This variation in their susceptibility is related to the amount of cell wall β-1,3-glucan, that the caspofungin target, which is more pronounced in the mycelial than in the yeast phase of the fungi. These results are interesting and new studies in the use of this drug in the treatment of PCM should be made, especially in studies using combinations of caspofungin with other antifungal drugs to increase their inhibitory capacity.

There are not many reports in the literature about resistant yeasts of *Paracoccidioides* spp. to antifungal therapies. There is a study however, that relates clinical and *in vitro* resistance to ketoconazole and trimethoprim-sulfamethoxazole. In this study, they have found that patients infected with *P. lutzii* had good responses to trimethoprim-sulfamethoxazole, while those infected with *P. brasiliensis* relapsed with the same

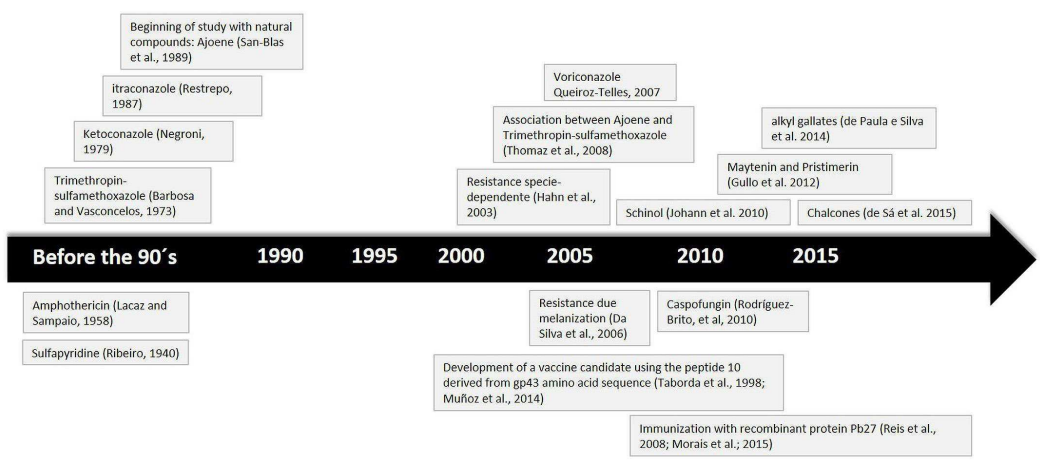

FIGURE 3 | Time line based on studies that brings development and new insights on treatment and active biomolecules against PCM.

drug administration (Hahn et al., 2003). In a different way, another study verified that the melanization process decreased susceptibility to antifungal agents, particularly amphotericin B, what can lead to resistance (da Silva et al., 2006).

Due to these facts, new drugs that are safer, more effective, cheaper and with shorter periods of therapy, seem warranted for the treatment of PCM. In this sense, many groups have been developing new alternatives treatment.

One trend is the study of natural and semi-synthetic compounds with great biological activity. In 1989 there began the evaluation of the antifungal activity of Ajoene, a compound derived from ethanolic garlic extracts. These inhibited the growth of *P. brasiliensis* by affecting the integrity of the fungal cytoplasmic membrane (San-Blas et al., 1989). The same authors discussed the possible involvement of ajoene on the sulphydryl metabolism of *P. brasiliensis*, inhibiting the effect on the yeast cells but not on the mycelial cultures (San-Blas et al., 1993). Alterations was observed in phospholipid, fatty acid proportions, phosphatidylcholine, and phosphatidylethanolamine in both phases and reduced saturated fatty acids in the yeast phase, with a corresponding increase in the unsaturated components (San-Blas et al., 1997). Two studies evaluated the antifungal effect of the ajoene in murine models were published; one showing a significant reduction in the levels of antibodies at the 10th week of treatment (Maluf et al., 2008). The other showed a positive additive effect when ajoene therapy was used in association with antifungal drugs (sulfametoxazol/trimethoprim) and a protective proinflammatory immune response (Thomaz et al., 2008).

Martins et al. (2009) showed that *Paracoccidioides* spp. isolates were susceptible to curcumin, a compound produced by the rhizome of *Curcuma long*, and which presented more inhibition effect than the antifungal agent, fluconazole. Johann et al. (2010a) found that the extract from *Schinus terebinthifolius* presented strong antifungal activity against *P. brasiliensis* isolates. Another study from the same authors showed that two compound isolates from the extract of *Schinus terebinthifolius*, schinol, and a new biphenyl compound, had antifungal activity against *P. brasiliensis* isolates. Schinol presented a synergistic effect when combined with Itraconazole (Johann et al., 2010b).

The 6-quinolinyl and quinolinyl N-oxide chalcones, specifically those named 4c and 4e, presented strong activity against *P. brasiliensis*. Histopathological analysis and a progression score of the disease in mice showed that the 4c compound was able to control inflammation and resolved the infection with better results than treatment with Itraconazole and 4e, while avoiding granuloma formation and preservation of lung tissue (de Sá et al., 2015).

Gullo et al. (2012) evaluated natural and semi-synthetics compounds such as maytenin and pristimerin and observed excellent minimum inhibitory concentration against different isolates of *P. brasiliensis*. In the same way, de Paula et al. (2014) evaluated the antifungal activity of the Alkyl gallates, which presented important biological activity reported by the literature, against different fungi species, including different isolates of *P. brasiliensis* and *P. lutzii*. They observed that these molecules presented important relations between the structure and activity, and that the decyl gallate have special activity against *Paracoccidioides* species.

Clinical and experimental data indicate that cell-mediated immunity plays a central role in host defenses against infection by *P. brasiliensis*, whereas high levels of specific antibodies and polyclonal activation of B cells are associated with more severe forms of disease (Cano et al., 1998).

The gp43 contains epitopes capable of producing a cellular immune response in guinea pigs (Rodrigues and Travassos, 1994) and human patients (Saraiva et al., 1996). The sensitivity of the immune response in mice to gp43 occurs by proliferation of CD4 + Th1 (Travassos et al., 1995). These epitopes stimulate CD4 + Th1 lymphocytes, which produce interferon (IFN-γ), which has the function of stimulating the formation of granulomas that may contain yeasts (Brummer et al., 1988). However, the contribution of each subtype of T cell in the immune response of the host

depends on the genetic patterns and an immunity with a balance of CD4/CD8 regulating the secretion of cytokines of the Th1 and Th2 type, which correlates with resistance of the host to infection by *P. brasiliensis* (Chiarella et al., 2007).

A 43 kDa glycoprotein (gp43) has 416 amino acids, where a specific stretch of 15 amino acids designated as P10, is recognized by T lymphocytes in mice and humans. The protective effect of P10 is related to inducing an immune response of Th1 dependent IFN-γ- dependent on isogenic mice (Taborda et al., 1998).

Isogenic mouse strains immunized with P10, developed lung infection 200 times less intense than the unimmunized animals (Taborda et al., 1998). Iwai et al. (2003) using TEPITOPE software verified the probability of caucasian HLA-DR recognizing different peptides. They verified that P10, a promiscuous peptide, was an important vaccine candidate for use in humans (Iwai et al., 2003). This peptide could be associated with drugs commonly used for the treatment of PCM and presented an additive effect in the experimental model using BALB/c mice. This demonstrates the capacity of peptide P10 to be useful for reducing the treatment time of this mycosis (Marques et al., 2006). Besides this, Magalhães et al. (2012) demonstrated the potential use of primed dendritic cells (DCs) with P10 as a vaccine that can protect the host against the development of PCM or treating a well-established disease.

According to studies developed in the last years, innate immune system and DCs play an important role in the resolution of *Paracoccidoides* spp. and other dimorphic fungal infections (Thind et al., 2015). DCs play a crucial role in the detection of pathogens, trigger an initial response of the host, as well as instruction to the adaptive immune response. It is also known that DCs play an important role in the induction of effector T cells that *P. brasiliensis* infection control and has been shown that *P. brasiliensis* induces regulatory DCs in susceptible mice. This, in turn, promotes IL-10 production and contributes to the infection susceptibility (Ferreira et al., 2007). Recently, Dos Santos et al. (2011) demonstrated that *P. brasiliensis* infection stimulates migration of DC and, bone marrow-derived DC, when stimulated by *P. brasiliensis,* migrate to the lymph nodes and activate a T-cell response. These studies open up new perspectives since the understanding of the regulation of the DC migration allow for the development of tools to efficiently activated a T-cell response aiding in the control of PCM.

The latest research about alternative therapies presented the immunization in murine models with rPb27, a recombinant protein of *P. brasiliensis,* showing its protective effect against the PCM and its important ability to prevent pulmonary fibrosis (Reis et al., 2008; Fernandes et al., 2011a,b; Morais et al., 2015).

The **Figure 3** we present a time line based on studies that bring development and new insights into the treatment and use of active biomolecules against PCM.

The treatment of mycosis is a great challenge for science, and in the PCM, this is not different. The problem is even more challenging because since 2006, no antifungal was approved. Of the drugs available today, many cannot be orally administered, have high toxicity, display cases of resistance, and present drug interactions. Thus, the development of new antifungal therapies has become an increasingly challenging problem mainly because of their growing resistance. This issue leads to a search for new antimicrobial agents that have different mechanisms to effectively combat infections and that do not contribute to the resistance of the pathogens that may complicate any therapy (Krachler and Orth, 2013).

CONCLUSION REMARKS

The advances in PCM studies bring us a better knowledge of how the interaction with the host was constructed during its evolution enabling the fungi to evade from host human immune system and remain in the organisms causing a mycosis with a high incidence in Latin America. This disease is a great public health issue that, with agricultural expansion, has an increasing occurrence area that may affect many more people in the future. This expansion is an alarming problem since the detection of the disease is difficult depending on the isolate, the patient, and the fact that the treatment of the PCM is difficult given the limited arsenal available against it.

The studies we present in this review are evidence of a great effort in the search for knowledge of the PCM and its etiologic agents, *P. brasiliensis* and *P. lutzii,* in the last years. The details of the *Paracoccidioides*-host interaction, the advances in the use of animal models to study the disease, and the discovery of new treatment methods and anti-*Paracoccidioides* agents, reveal a promising future in combating this disease.

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Searching new antifungals: The use of *in vitro* and *in vivo* methods for evaluation of natural compounds

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ABSTRACT

In the last decades, the increased number of immunocompromised patients has led to the emergence of many forms of fungal infections. Furthermore, there are a restricted arsenal of antifungals available and an increase in the development of resistance to antifungal drugs. Because of these disadvantages, the search for new antifungal agents in natural sources has increased. The development of these new antifungal drugs involves various steps and methodologies. The evaluation of the *in vitro* antifungal activity and cytotoxicity are the first steps in the screening. There is also the possibility of antifungal combinations to improve the therapy and reduce toxicity. Despite that, the application of the new antifungal candidate could be used in association with photodynamic therapy or using nanotechnology as an ally. *In vivo* tests can be performed to evaluate the efficacy and toxicity using conventional and alternative animal models. In this work, we review the methods available for the evaluation of the antifungal activity and safety of natural products, as well as the recent advances of new technology in the application of natural products for antifungal therapy.

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Contents

1. Introduction . . . 69
2. *In vitro* Methods . . . 69
   2.1. AGAR diffusion . . . 69
   2.2. Macrodilution and microdilution assay . . . 70
   2.3. Minimum fungicidal concentration assay . . . 70
   2.4. Colorimetric assays . . . 70
   2.5. Cytotoxicity assay . . . 70
   2.6. Combination of substances . . . 71
3. *In vivo* models . . . 71
   3.1. Toxicology . . . 71
   3.2. Pharmacokinetics . . . 71
   3.3. Alternative animal models . . . 72
   3.4. Efficacy in mammalian model . . . 72
4. New strategies to enhance the efficacies of antifungals . . . 73
   4.1. Photodynamic therapy (pdt) using natural compounds . . . 73
   4.2. The development of antifungal natural products using nanoparticle technology . . . 73
   4.3. Combination of PDT and nanoparticles of natural compounds against antifungal infection . . . 74
5. Conclusion . . . 75
Acknowledgments . . . 75
References . . . 75

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1. Introduction

While the availability of antifungal agents is restricted, the similarity between fungal and mammalian cells is the main reason for the elevated toxicity frequently associated with antifungal therapy (Ostrosky-Zeichner et al., 2010; Pierce et al., 2013). Despite the discovery of new molecules and the new formulation availability to reduce the toxicity and increase bioavailability, the search for new antifungal agents and the characterization of novel targets are a continued need (Agarwal et al., 2008; Martinez-Rossi et al., 2008; Wiederhold and Patterson, 2015). An ideal antifungal agent should have a broad-spectrum and minimum side effects (Carrillo-Muñoz et al., 2006). Plants, animals, and terrestrial or marine microbes are good sources for potential antifungals (Cruz et al., 2007; Rajeshkumar and Sundararaman, 2012; Rojas et al., 2006) because of their extensive biosynthetic capacity. This makes them a precious source of therapeutic compounds (Schmidt et al., 2008). Essential oils, flavonoids, alkaloids, proteins, peptides, glycoproteins (Satya et al., 2005), and tannins are found in plants and can be employed as models for the synthesis of new compounds (Newman and Cragg, 2007). In recent decades, about a quarter of the drugs used worldwide have been discovered in natural products or their derivatives (Balunas and Kinghorn, 2005). Most of the antifungal drugs used today in fact were discovered from natural sources. For example, amphotericin B, a polyene, was discovered in the 1950s from *Streptomyces nodosus* cultures (Trejo and Bennett, 1963). Micafungin is an antifungal drug derived from the fungi *Coleophoma empetri* (Jarvis et al., 2004) and caspofungin is obtained from a fermentation of the fungi *Glarea lozoyensi*. Both are antifungal drugs belonging to the echinocandin class (Abruzzo et al., 2000). In this study, we will review the *in vitro* and *in vivo* methodologies for the evaluation of efficacy and safety of natural products with antifungal potential. Fig. 1 represents all of the stages that a natural product should reach in order to be a candidate for use as an antifungal drug.

2. *In vitro* Methods

The search for natural compounds with antifungal activity has increased, however, the testing methods present many challenges. The diversity of testing methods and the lack of clearly defined testing conditions such as inoculum size and medium type, can lead to low reproducibility. To insure that the selection of compounds with relevant pharmacological action are worthy of following up, it is necessary that the selection and validation of primary screening assays be rapid, simple, and easy to implement. They must also produce quick results at a low cost. In this sense, the standardization of the *in vitro* antifungal susceptibility test has advanced in recent years. The Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing have set the benchmark methodology by providing laboratory tested, reproducible, and peer-reviewed standards to commercial antifungal drugs (EUCAST, 2008; M27-A3, 2008; M38-A2, 2008). Many authors have followed these documents in their studies (Alastruey-Izquierdo et al., 2015; Cuenca-Estrella et al., 2010; Cuesta et al., 2010; Liu et al., 2007) and were adapted for plant extracts (Scorzoni et al., 2007; Svetaz et al., 2010). Independent of the susceptibility test adopted, it is necessary to use a reference drug to validate the methodology. There are several drugs available for this purpose and the choice depends on the fungi (Cos et al., 2006).

2.1. AGAR diffusion

Agar diffusion technique is a semi-quantitative assay that consists of applying an amount of a sample with a known concentration (the plant extract, fraction of the extract or the pure substance) on the agar surface which previously was inoculated with a standardized amount of fungal cells. The sample can be applied by different techniques, such as disk diffusion, in which disks made of sterile filter paper (6 mm) are impregnated with the sample and then applied to the agar surface. The plate-hole, another agar diffusion technique, which the sample are placed into wells made in the agar medium (Hayhoe and Palombo, 2013; Rios et al., 1988). In both techniques, the sample diffuses into the agar medium, creating a circular concentration gradient. The yeast will then multiply and if the sample has antifungal activity, a growth inhibition zone will appear around the disk. This inhibition zone is measured in millimeters, and some authors classify it into three categories: total inhibition, partial inhibition, or no inhibition (de Souza et al., 2004; Kalemba and Kunicka, 2003).

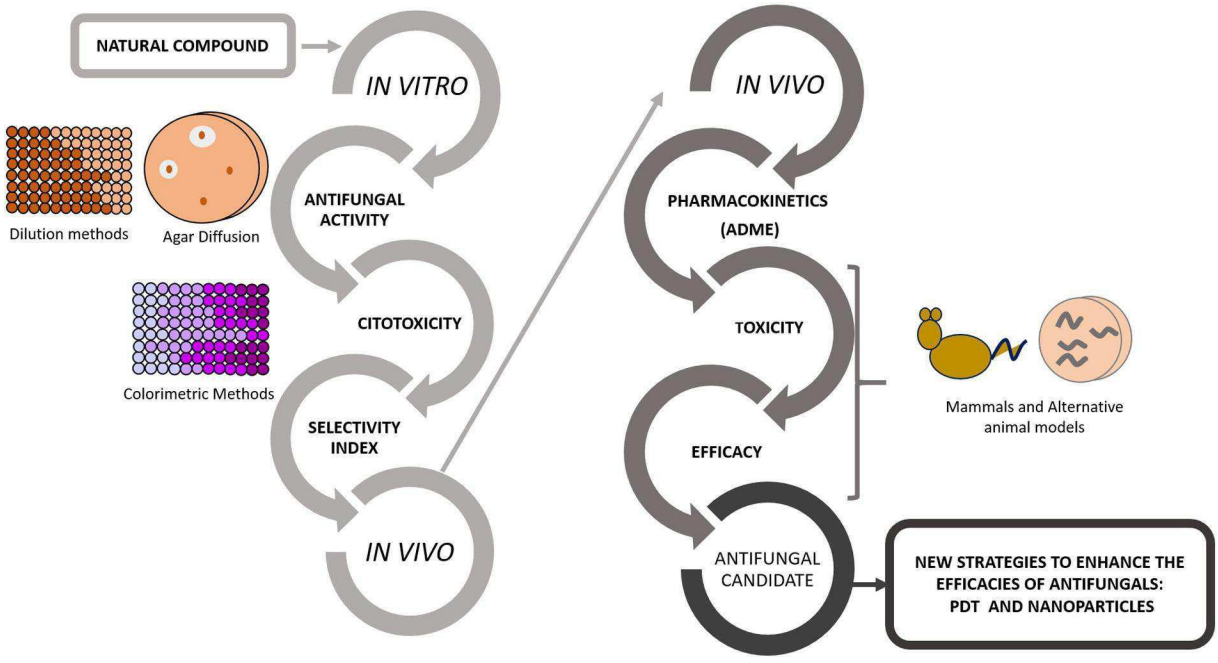

Fig. 1. Representative schema of the steps and assays for an antifungal drug candidate.

Many studies employed agar diffusion during the screening of antifungal activity which give semi-quantitative data, and before using more precise techniques like macrodilution (Kosalec et al., 2013) or microdilution (Ergin and Arikan, 2002).

The disadvantage of agar diffusion methodology is the use of large amounts of plant extracts; moreover, the lipophilicity, volatilization, amount of the compound, the agar media type, pH, and the volume of agar can all strongly affect the inhibition zone (Pauli, 2006). These factors in the agar diffusion technique must be carefully measured for consistency so as to avoid false-positive or negative results. In our group, Scorzoni et al. (2007) evaluated the antifungal activity of crude extracts, fractions, and pure substances from different species of the plant families by two different methodologies: agar diffusion and broth microdilution against *Candida* spp. and *Cryptococcus neoformans*, and observed that the microdilution method was more sensitive than the agar diffusion and moreover provided quantitative data.

2.2. Macrodilution and microdilution assay

The broth dilution methods are the most widely used assays to determine the antifungal activity. The documents standardized to yeasts (M27-A3, 2008) and filamentous fungi (M38-A2, 2008) from CLSI, describes the macrodilution and microdilution susceptibility test to antifungal drugs. These methods provide quantitative data about the Minimum Inhibitory Concentration (MIC) of the compound, which is the lowest compound concentration able to inhibit the antifungal growth.

The microdilution assay is recommended for the screening of natural compounds because of the high throughput potential, considerable savings in media usage, and requirement of a small amount of sample; moreover this method could be applied for different microorganisms (de Melo et al., 2013; Djouossi et al., 2015; Gehrke et al., 2013; Johann et al., 2010; Njateng et al., 2015; Pozzatti et al., 2008). The employment of the macrodilution technique to evaluate natural products as antifungals was reported in different studies (Bouzabata et al., 2013; Cabral et al., 2012; Pinto et al., 2013).

Interestingly, these tests have been standardized to antifungal drugs which suggest that it is necessary to optimize test conditions in order to adapt and standardize these for natural compounds. Our group used the microdilution methodology to demonstrate the antifungal activity of alkyl gallates, which are modified substances derived from natural compounds. The alkyl gallates were tested against several species of pathogenic fungi (*Candida* spp., *Cryptococcus* spp., *Trichophyton* spp., *Aspergillus* spp., *Paracoccidioides* spp., and *Histoplasma capsulatum*). These compounds showed great antifungal activity against most isolates and had a particularly good selectivity index (SI) value, making this compound an excellent candidate for a broad-spectrum antifungal prototype. This also is encouraging for the continuation of subsequent studies for the discovery of its mechanism of action (de Paula e Silva et al., 2014).

2.3. Minimum fungicidal concentration assay

By using both dilution methods, it is possible to determine if the substance is active, however it is not possible to determine if the compound will kill or just inhibit the fungal growth. For this purpose, the minimum fungicidal concentration (MFC) assay is performed. Small aliquots from each of the broth dilution tests are subcultured on a rich solid medium and incubated for a determined time and temperature, depending on the fungal species being tested. According to the documents standardized by the CLSI, the MFC is considered the lowest concentration of the substance in which no visible growth of subculture occurs. Moreover, MFC could give information about fungicide or fungistatic activity. If the MFC is the same as the MIC, it is considered to be a compound fungicide, but if the MFC is higher than the MIC, then it is a fungistatic (M27-A3, 2008). Our group studied the antifungal activity of maytenin and pristimerin against *Candida* spp., *Cryptococcus* spp., *P. brasiliensis*, *H. capsulatum*, *Aspergillus* sp. and *Trichophyton* sp. in which MIC values for the substances ranged from 0.12 to 250 $mg \cdot L^{-1}$. Maytenin demonstrated fungicidal activity for most of the yeasts tested (Gullo et al., 2012).

2.4. Colorimetric assays

The microdilution technique is recommended since that is less expensive (uses less media, inoculum, and less of the compound), and provides highly reproducible results. Fungal biomass is assessed visually by grading turbidity, or spectrophotometrically by measuring optical density. However, some species do not generate enough fungal biomass. Alternatively, biomass can be determined colorimetrically in which the fungal metabolic activity and the fungal biomass can be estimated using a colorimetric substance (Meletiadis et al., 2001a, 2001b). In this sense, colorimetric microdilution has been used to screen compounds with antifungal activity, as it was published by Liu et al. (2007) and Monteiro et al. (2012).

Meletiadis et al. (2001a) used the heterocyclic organic compounds, tetrazolium salts MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) and the XTT (sodium3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-itro)benzene-sulfonic acid hydrate) for antifungal susceptibility testing. These salts can penetrate rapidly into the cells and the dehydrogenase activity reduces to violet formazan crystals. This reduction is made by receiving electrons enzymatically from substances of the hydrogen transport system or non-enzymatically from artificial electron transporters (phenazine methosulfate and menadione). The amount of these crystals is quantified by spectrophotometrically estimating the number of living cells in the sample (Denizot and Lang, 1986; Freimoser et al., 1999).

The use of MTT results in non-water-soluble crystals and XTT is converted into a water-soluble crystal. However it needs the presence of an electron-coupling agent (Kuhn et al., 2003). Due to the product from XTT being water soluble, it is possible to study the biofilm metabolic activity with or without treatment measuring the supernatants (Pierce et al., 2008). The MTT method can be exemplified in *Aspergillus niger*, *Candida albicans* and *Trichophyton rubrum* studies (Fu et al., 2010). The XTT method is commonly used in biofilm formation and anti-biofilm activity of *Candida* spp. (Ansari et al., 2013; Ramage et al., 2012; Saharkhiz et al., 2012).

Another colorimetric method is the MABA (Microplate Alamar Blue Assay), alamarBlue®, in which resazurin is the active substance. For example, Liu et al. (2007) and Monteiro et al. (2012) studies demonstrate the importance of this method in the screening of antifungal natural compounds. Antifungal susceptibility tests to *Paracoccidioides* spp. were evaluated using the microdilution technique according to the document M27-A3 (2008). MABA was necessary in order to have reliable, reproducible, quick, and highly accurate routine laboratory tests (de Paula e Silva et al., 2013).

2.5. Cytotoxicity assay

Once the antifungal activity of a compound of interest is established, it is necessary to know how much of this compound can influence the viability of mammalian cells using an *in vitro* cytotoxicity assay. Cellular death, changes in cell membrane permeability, and metabolism inhibition are characteristics of cellular toxicity. Cell viability tests can be easily performed with *in vitro* cellular systems and vital dyes. Some of these systems were described in the above sections (Rogero et al., 2000). The selection of a cytotoxicity assay relies on the performance and quality of the data produced. The alamar blue and MTT assays are high quality and are suitable to identify cytotoxic compounds; however, the alamar blue assay is homogenous and provides the advantage of higher sensitivity through which it may detect low cell densities. It is

recommended to carefully consider the possibility of false positives and negatives due to the inhibition or induction of drug metabolizing enzymes, as this could lead to a misinterpretation of the data (Hamid et al., 2004).

Sulforhodamine B is another method widely used whose mechanism is based on the labeling of overall protein content within the cells that were not killed with the substance tested (Rubinstein et al., 1990). The amount of dye from pigmented cells is directly proportional to the total mass of protein and thus is correlated with the number of cells (Papazisis et al., 1997; Vichai and Kirtikara, 2006). Some compounds interfere in the MTT reduction with no effect in cell viability. This does not occur with sulforhodamine B which is based on cell density determination unlike MTT which requires cellular metabolic activity to convert colorless tetrazolium to purple colored formazan dye. In addition, another benefit of sulforhodamine B is its linearity.

Many studies use the MTT method to perform cytotoxicity with medicinal plants like *Breonadia salicina* (Mahlo et al., 2013), *Maytenus undata* (Mokoka et al., 2013); *Retama raetam* (Edziri et al., 2012); *Ricinus communis* (Zarai et al., 2012); *Asteraceae* plant family (Zapata et al., 2010), and *Tanacetum balsamita* (Yousefzadi et al., 2009). Other studies use sulforhodamine B, like *Rudbeckia laciniata* (Lee et al., 2014) and *Pleurospermum kamtschaticum* (Lee et al., 2012).

The IC50 can be defined as a substance concentration that can inhibit 50% of the cells. This value can be used to find also the selectivity index (SI), which is the ratio between the IC50 and MFC values. The most important index is the value of SI: the activity of the compound against the organism is better and presents lower cytotoxicity, thus, the safety of the compound is greater (Protopopova et al., 2005). These parameters were evaluated in many studies in the search of antifungal activity (Ghoneim et al., 2013).

2.6. Combination of substances

The combination of substances is a promising way to increase the percentage of successful treatments (Shao et al., 2007). Combinations of two or more antifungals and combinations of antifungal drugs with natural products have been evaluated in several studies by an *in vitro* method called “checkerboard” that is widely used (Greco et al., 1995; Odds, 2003).

The checkerboard test was based on the procedure established by CLSI for the broth microdilution technique for antifungal susceptibility testing. Two compounds are combined in 96 plate wells, placing one of them in descending concentration in rows and the other in columns, forming the combination of the compounds. To check the combinatorial effect of the compound, the fractional inhibitory concentration index (FICI) was adopted, which can be synergistic, additive, indifferent, or antagonistic. The FICI is calculated as the sum of the fractional inhibitory concentration (FIC) of the compound 1 + FIC of compound 2. The FIC of compound 1 is obtained by dividing the MIC when it is used in combination by the MIC of one compound by itself. The same calculation is performed for compound 2. The combination is defined as synergistic when the FICI is equal or less than to 0.5; additive FICI is greater than 0.5 and smaller/equal to 1.0; FICI is indifferent when greater than 1.0 and smaller/equal to 4.0; and antagonistic if FICI is greater than 4.0 (Clancy and Nguyen, 1997; Johnson et al., 2004; Odds, 2003; Patterson et al., 2000). The combination of antifungal drugs and natural compounds is described for pathogenic fungi (Menezes et al., 2012; Rodero et al., 2000; Venturini et al., 2011). In a study conducted in our laboratory evaluating the alkyl protocatechuates combined with fluconazole against *T. rubrum* and *Trichophyton mentagrophytes*, the heptyl protocatechuate showed a synergistic activity, reducing the MIC of fluconazole in fourfold (Soares et al., 2014).

3. *In vivo* models

3.1. Toxicology

To develop new antifungal compounds it is necessary to take into consideration its safety. For that, it should be considered for *in vivo* tests in mammals, lower vertebrates and invertebrates. Vertebrate models are usually used instead of invertebrates due to their closer resemblance of their bodily structure, function, and metabolism to that of humans. However, invertebrate models have been utilized due to low rearing costs, well-defined genetic backgrounds, and because they are easy to manipulate (Dolganiuc and Szabo, 2009).

The toxicity analysis in mammals such as mice and rats include the administration of certain dosages of the compound studied and lethal dose (LD50) can be calculated. Furthermore, loss of body weight and other signs of significant toxicity are continuously monitored. Finally, histological analysis of damage to organs and biochemical liver and kidney function tests can be performed. Concerning this, some studies have evaluated the acute and/or subchronic toxicity of natural products with potential antifungal activity. In one study of acute toxicity, Li et al. (2012) showed that the LD50 of a natural product isolated from *Pogostemon cablin* was much higher than the daily dose administered for the efficacy experiments for the treatment of *Candida* infections. Fontenelle et al. (2007) performed another experiment of acute and subchronic toxicity of the essential oil of *Lippia sidoides* in rats in which they observed no alterations in the organs, serum biochemical parameters, or body weight. Moreover, the administration of the essential oil of *Psidium cattleianum* at doses of 100, 200, and 500 mg.$\cdot kg^{-1}$ did not cause alterations in body weight or death of any animal (Castro et al., 2015). On the other hand, the acute and subacute oral treatment of a hexane extract of *Pterocaulon polystachyum* was relatively toxic to the liver and kidney (Regner et al., 2011).

The use of alternative animal models was also used in toxicology. Regarding the lower vertebrates, the zebrafish (*Danio rerio*) has been used as a toxicity model. Zebrafish should be used in studies of acute and chronic systemic toxicity and specific organ toxicities (Scholz, 2013). For the assay, the zebrafish embryos are incubated in 48-plate wells containing 1 ml of test medium per well and are exposed to different concentrations of compounds. From this data, histological analysis can be conducted (Driessen et al., 2013). Zebrafish embryos were used as a toxicity model for pahayokolide A, a compound from *Lyngbya* which has antifungal and antibacterial activities. However, the compound was acutely toxic to zebrafish embryos, killing 100% of embryos at concentrations of 5 mg$\cdot L^{-1}$ or higher (Berry et al., 2004).

In modern studies, in the evaluation of toxicity of new compounds, invertebrate models also show suitable application for pre-screening. A preliminary assessment of toxicity in *Galleria mellonella* reduces the probability of an agent with *in vitro* activity advancing to mammalian models and failing. This alternative model could detect the toxic effect of the compound earlier saving time and money while reducing the number of mammals required (Desbois and Coote, 2012). The nematode *Caenorhabditis elegans* (Boyd et al., 2012) also should be used for screening toxicity of new antifungal compounds. Breger et al. (2007) observed that in the *C. albicans–C. elegans* model, fluconazole was effective in prolonging survival of nematodes up to a concentration of 32 mg$\cdot L^{-1}$; however at higher concentrations (100 mg$\cdot L^{-1}$) the drug was toxic to the nematode.

3.2. Pharmacokinetics

After having observed that the natural compound has satisfactory antifungal activity *in vitro*, it is necessary to conduct tests to evaluate the processes of absorption, distribution, metabolism, and excretion (ADME) of the compound in the organism. These processes are investigated by pharmacokinetics, which include *in silico*, *in vitro*, and *in vivo* strategies.

The *in silico* models use computational tools such as VolSurf, which predict human intestinal absorption, protein binding, blood–brain barrier permeation, drug solubility, metabolic stability, and volume of distribution (Koukoulitsa et al., 2005). In this context, the VolSurf computational method was used to investigate the pharmacokinetic profile of antimicrobial compounds (Karioti et al., 2011).

Recently, *in vitro* studies with ADME have been incorporated earlier in the drug discovery phase. In assays for intestinal drug permeability, Caco-2 cells, human colon carcinoma cell line, is a widely used model. The experiment is performed in a 24-well transwell, in which measurements are made of the uptake of compounds from the lumen (inside the transwell) to the blood (outside the transwell). Additionally, the interaction of the compound with efflux pumps and metabolizing enzymes could be evaluated in the Caco-2 cells. Drug metabolism has also been estimated for *in vitro* systems, which include microsomes and hepatocytes. Microsomes are endoplasmic reticulum membrane vesicles prepared from the liver, which contains the P450 isoforms and one of the phase II conjugating enzymes, UDP-glucuronosyltransferase (UGT). Hepatocytes have all the hepatic drug-metabolizing enzymes and cofactors. Therefore, they are used to study both phase 1 and 2 liver drug metabolism pathways. Drug screening using microsomes or hepatocytes involves the incubation of the drug candidate in a 96-well plate, followed by quantification of the amount of the parent compound that remains after metabolism. Moreover, test compounds are incubated with microsomes or hepatocytes in the presence of various isoform-specific substrates. Thus, it is possible to identify the cytochrome P450 (CYP) isoforms that are involved in the metabolism of the compound, as well as determining whether the metabolites generated are more active or more toxic than the compound. Furthermore, it is possible to evaluate natural compound–drug interactions (Li, 2001; Li, 2005; Mazzari and Prieto, 2014).

The effect of antifungal ketoconazole was studied in Caco-2 cells, as well as the involvement of P-glycoprotein with this drug. The basal-to-apical transport of rhodamine 123, a P-glycoprotein substrate, was inhibited by ketoconazole, yet inversely, the apical-to-basal transport of rhodamine 123 was increased by this drug. Therefore, ketoconazole could interact with P-glycoprotein mediated transport. Moreover, it was demonstrated that P-glycoprotein was not involved in the transport of ketoconazole in the intestinal epithelia (Takano et al., 1998).

With regard to the *in vitro* metabolic profile, an antifungal piperazine propanol derivative was evaluated by incubation with microsomes of several species and with rat hepatocytes. In hepatocyte incubations, several epoxide- and hydroxylated metabolites could be observed. Additionally, these metabolites formed several glucuronides. From the analysis of the metabolic pattern in microsomes, products of carbamate hydrolysis were characterized. This hydrolysis was highly species dependent (Wind et al., 2009).

The pharmacokinetic profile also could be investigated in the animal models. These studies may be done by the administration of different dosages and in a second step, the quantification of the compounds and their metabolites by high performance liquid chromatography. Thus, parameters such as maximum serum concentration (Cmax), area under the curve of plasma concentration versus time (AUC0–t), and half-life (t1/2) can be determined. In this context, Li et al. (2012) administered a natural product isolated from *P. cablin* in mice and a pharmacokinetics profile was determined. The compound was effective against *Candida* spp. *in vitro* and *in vivo* and the pharmacokinetic parameters of the compound revealed that it was easily absorbed after oral administration in mice with the Cmax values higher than the corresponding MIC. Moreover, the mean AUC increased proportionally with the dosage, which was consistent with the dose-dependent effect of the natural product on the fungal load in the vagina. However, the compound suffers from a rather short T1/2 (approximately 51–53 min), which indicates that more frequent administration is required to obtain the optimum therapeutic response. The results suggest that a natural product might be a good candidate for the development of new anti-*Candida* agents.

The comprehension of the cited models should be considered, because in addition to pharmacological properties, good pharmacokinetic and toxicological profiles are crucial determinants of the ultimate clinical success of natural products as new antifungals.

3.3. Alternative animal models

In vitro experiments give us the basic knowledge about the fungal cells directly in contact with the compound. However, after determining the antifungal activity of the compound, it is necessary to understand if this compound has the same activity when it is submitted to complex systems, where degradation and/or modification of the compounds can occur and change its potential. Considering this, *in vivo* experiments are crucial for the confirmation of in vitro data regarding the antifungal compound candidate.

Despite the need for using animal models to perform studies, currently the use of mammalian animal models is highly regulated with much dependence on ethical committees and good laboratory practices. In 1959, Russell and Burch proposed the theory of the three Rs (Reduction, Refinement and Replacement) aiming to make the world aware of this scientific issue in order to compel them to search for alternatives to the use of traditionally used (but highly regulated) experimental animal models (Arora et al., 2011).

The moth, *G. mellonella* and the nematode worm, *C. elegans* are good examples of such alternative animal models (Desalermos et al., 2012; Fuchs and Mylonakis, 2006; Mylonakis et al., 2007). The possibility to incubate larvae at temperatures ranging from 25 to 37 °C can simulate conditions of fungal infection in mammals while allowing the possibility to administer the exact quantities of pathogens/drugs. These are important advantages of *G. mellonella* as a fungus-host model. Moreover, *G. mellonella* has six types of cells, some of which have phagocytic capacity. This plays an important role in their defense system. Furthermore, cell density in the haemolymph changes during infection and can be easily measured and used as a parameter of the response of larvae after exposure to pathogens (Desalermos et al., 2012; Fuchs and Mylonakis, 2006; Fuchs et al., 2010).

Concerning *G. mellonella*, the efficacy of antifungal drugs show a good relation between *in vitro* test and *in vivo* treatment of the larvae infected with *C. albicans*, *C. krusei* and *C. tropicalis* (Mesa-Arango et al., 2013; Scorzoni et al., 2013). The efficacy of anthraquinones from a Rubiaceae plant, *Morinda tomentosa*, against *C. albicans* was proven in the *G. mellonella* model, however these compounds did not show significant antifungal activity in the model (Favre-Godal et al., 2014).

Regarding *C. elegans*, genomic knowledge enables an understanding of fields not well elucidated. This is one of the main advantages of this model (Pukkila-Worley et al., 2011). *C. elegans* was suitable to evaluate libraries of chemical compounds for antifungal activities for *C. albicans* and also for screening toxic compounds (Breger et al., 2007; Pukkila-Worley et al., 2009). Recently, the antifungal activity of magnolol and honokiol from the plant *Magnolia officinalis* were also evaluated *in vivo* against *C. albicans* using *C. elegans* (Sun et al., 2015). Thus, the use of alternative animal models is useful in the search for new natural antifungal compounds while avoiding obstacles in current antifungal research such as cost and time constraints.

3.4. Efficacy in mammalian model

The mammalian animal models are very important in the search for new treatments with mice being the most widely used models. The choice of animal species along with the route of inoculation of the organism depends on the desired goal. The intraperitoneal route is widely used due to the ease of manipulation. The intravenous route is a standardized technique and provides answers about the spread of the organism to different organs. The intracerebral approach allows

for the study of the proliferation of the yeast in the central nervous system. Intranasal and intratracheal routes are best utilized to mimic natural respiratory infections while also allowing for the evaluation of the ability of the organism to spread from the lungs. The infections in these animals may be subclinical or lethal depending on the size of the inoculum, the route of inoculation of the strain in question, as well as the strain and immune status of the animal (Casadevall and Perfect, 1999; Dixon and Polak, 1986).

Using mammal models, it is possible to evaluate the survival rate or death of animals along with their fungal burden in different organs. Consequently, it is possible to analyze the effectiveness of the immune system of the host, the virulence of the microorganism, the effectiveness of treatment with antifungal drugs, and comparing the host response to different strains of the microorganism (Chrétien et al., 2002).

In vitro and *in vivo* studies to evaluate the synergistic effect of the combination between new compounds and antifungal drugs have also been performed. The combination of berberine and amphotericin B allowed the survival of mice with disseminated candidiasis for 22 more days compared with the amphotericin B treatment alone (Han and Lee, 2005). Additionally, the combination of amphotericin B with grape seed extract has promising results as shown by Han (2007). The antifungal activity of the extracts of the *Combretum* and *Terminalia* species against *C. albicans*, *C. neoformans*, *Microsporum canis* and *Sporothrix schenckii* was evaluated by Masoko et al. (2010) and verified activity in mice with wounds infected with fungi by topical administration. Eight saponin isolates from *Tribulus terries* were also tested by Zhang et al. (2005) against *Candida* species and *in vivo* results showed potential antifungal activity. The activity of pogostone, a substance isolated from *P. cablin*, was evaluated by Li et al. (2012) *in vitro* and *in vivo* against clinical isolates of *Candida* spp.

4. New strategies to enhance the efficacies of antifungals

4.1. Photodynamic therapy (pdt) using natural compounds

In photodynamic antimicrobial chemotherapy (PACT), antimicrobial photodynamic inactivation (aPI), or photodynamic therapy (PDT), a combination of a sensitizing drug or compound and visible light, cause selective destruction of microbial cells (Donnelly et al., 2008). This therapy combines a pharmacologically inert chromophore, termed as photosensitizer (PS), with a light that corresponds to the chromophore's specific absorption wavelength. The chromophore's exposure to its specific light wavelength in the presence of oxygen induces the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) radicals, including singlet oxygen and hydroxyl radicals (Silva et al., 2015). The generation of nitrosative and oxidative stresses by the process reduces the probability of the selection of resistant strains because they have diverse cellular targets. Also, these radicals cause alterations in the structure of the membrane and the fungal cell wall, providing penetration of the PS into the cell. At the same time, the radicals cause damage in cytoplasmatic organelles and nucleic acids, inducing cell death by apoptosis, necrosis, or autophagy (Baltazar et al., 2015b).

PDT was initially developed as a cancer treatment, but has also been investigated as a treatment for choroidal neovascularization secondary to age-related macular degeneration, localized infections, and for disorders related to dermatology and immunology. Many different types of photoactivable molecules have been synthesized and tested as PDT agents, including porphyrins, chlorins, bacteriochlorins and phthalocyanines (Agostinis et al., 2011).

For PDT to be both effective and safe, the PS should be delivered in therapeutic concentrations to the target cells and simultaneously be absorbed in only small quantities by non-target cells, minimizing undesirable side effects in healthy tissues (Huang et al., 2012).

In vitro methods using PDT have been widely performed against several species of microorganisms, including bacteria, viruses, protozoa, and fungi (Arboleda et al., 2014; Brooks et al., 1994; Choi et al., 2014; Lim et al., 2012; Lyon et al., 2011; Makarov et al., 2014; Morton et al., 2014; Rossoni et al., 2014; Sabino et al., 2015; Soares et al., 2011; Song et al., 2011; Takahashi et al., 2014). Silva et al. (2015) report that after cancer, infections represent the next most frequent application of PDT. Some natural compounds are being described as well-established photosensitizers. Gasparetto et al. (2010) evaluated the extracts of *Althernanthera maritima* (an herbaceous plant commonly found in the sandy beaches of the Brazilian coast), as a photosensitizer against *Candida dubliniensis*. It was concluded that hexane and ethanol extracts of *A. maritima* aerial parts, by laser irradiation at 685 nm, produced an antifungal effect against *C. dubliniensis*. Hypericin is a natural pigment of hypericum plants and is prominent among photosensitizers, exerting phototoxicity through several mechanisms (Theodossiou et al., 2009). The photodynamic effect of this natural pigment was evaluated against a species of *C. albicans*, *Candida parapsilosis*, *Candida krusei* (Rezusta et al., 2012) and also against the dermatophytes *T. rubrum* and *T. mentagrophytes* (Paz-Cristobal et al., 2014). The hypericin aPI was fungicidal against the dermatophytes when tested *in vitro*. Regarding *Candida* spp., only *C. albicans* could be effectively killed without damage to normal skin cells.

Previous studies reported the use of PTD in animal models against several species of fungi (Baltazar et al., 2015c; Chibebe Junior et al., 2013; Dai et al., 2011; Machado-de-Sena et al., 2014; Martins Jda et al., 2011; Mima et al., 2010; Mitra et al., 2011; Oberste-Lehn and Plempel, 1977). However, there are just a few reports of PDT in fungal infections in animal models using natural photosensitizers. Regarding natural compounds, Dovigo et al. (2013) described the photoinactivation of *C. albicans* in a murine model of oral candidiasis using curcumin as a PS. It was concluded that a single application of PDT was sufficient to eliminate *C. albicans* from mice that received 80 μM of curcumin and 37.5 J/cm of light.

4.2. The development of antifungal natural products using nanoparticle technology

The development of new formulations for antifungal compounds has been widely studied with the aim of reducing toxicity and increasing the bioavailability and consequently the efficacy. Nanotechnology is an alternative formulation providing interesting results with traditional and new antifungal compounds. Amphotericin B nanoparticles reduced the toxicity and maintained or increased the antifungal activity against *Candida* spp. (Casa et al., 2015; Tang et al., 2015; Tang et al., 2014). Similar results were observed with itraconazole nanoparticle (Qiu et al., 2015), fluconazole nanoparticles (Longhi et al., 2015), and miconaloze (Kumar and Poornachandra, 2015). Nanotechnology was also used for new natural antifungal candidates. Curcumin is known by a high diversity of biological activity, however the low aqueous solubility and poor bioavailability affects its use. The use of nanoparticles of Curcumin increase the antimicrobial activity of this compound against different microorganisms, including *Penicillium notatum* and *Aspergillus niger* (Bhawana et al., 2011). The allicin (active principle of garlic) in nanoparticle formulation showed significant reduction in the MIC for yeast and filamentous fungi (Luo et al., 2009). Copaiba oil has its antifungal activity enhanced after nanoencapsulation and was considered an alternative treatment for cutaneous infections caused by yeasts and dermatophytes (Svetlichny et al., 2015). *In vivo* study using a model of oral candidiasis, compared eugenol loaded in nanoparticles and liquid eugenol. The eugenol nanoparticles demonstrated higher capacity to reduce the log cfu (colony-forming unit) value than the liquid eugenol, showing an enhancement in antifungal activity when eugenol was formulated in nanoparticles (Garg and Singh, 2011).

4.3. Combination of PDT and nanoparticles of natural compounds against antifungal infection

The combination of nanotechnology and PDT is also a new feature to enhance the efficacies of antifungals. Lipid and detergent nanostructures like liposomes and micelles were routinely used in PDT before nanotechnology became a separate area of specialization. Today, the revolution of nanotechnology has provided many examples of nanoscale drug-delivery platforms, including liposomes, lipoplexes, nanoemulsions, micelles, polymer nanoparticles, silica nanoparticles, and fullerenes among others, that have been applied to PDT as described by Huang et al. (2012). Natural polymers such as proteins and polysaccharides have been studied for use in PDT. Furthermore, the use of chitosan, a biopolymer derivative from

Table 1
In vitro and *in vivo* methods and applications for screening of natural antifungal compounds.

Objective	Methods/models	Application-reference	Natural compound/drug
In vitro efficacy	Agar diffusion	Dzoyem et al. (2011)	*Diospyros canaliculata* extracts
		Palá-Paúl et al. (2012)	*Chamaecyparis lawsoniana* essential oils
		Galán et al. (2013)	Isoquinoline derivatives
	Macrodilution	Cabral et al. (2012)	*Juniperus communis* essential oils
		Pinto et al. (2013)	*Ferulago capillaris* essential oil
		Bouzabata et al. (2013)	*Myrtus nivellei* essential oil
		Gullo et al. (2012)	*Maytenus ilicifolia* substances
	Microdilution	De Melo et al. (2013)	*Lippia gracilis* essential oils
		Gehrke et al. (2013)	Compounds of *Schinus lentiscifolius*
		Djouossi et al. (2015)	Flavonoids
		Njateng et al. (2015)	Terpernoid saponin and other compounds
		De Paula e Silva et al. (2014)	Alkyl gallates
		Pozzatti et al. (2008)	Essential oils of different plants
		Johann et al. (2010)	Schinol and biphenyl compound of *Schinus terebinthifolius*
	Colorimetric assays (MTT, XTT and MABA)	Ansari et al. (2013)	Zizyphus spina-christi (Jujube honey)
		Fu et al. (2010)	Series of caffeic acid amide
		Liu et al. (2007)	Extracts of *Combretum* and *Terminalia*
		Monteiro et al. (2012)	Different natural extracts
		Saharkhiz et al. (2012)	*Mentha piperita*
		Ramage et al. (2012)	Tea tree oil and its derivate
	Synergism	Han and Lee (2005)	Berberin + AMB
		Han (2007)	Grape seed extract + AMB
		Menezes et al. (2012)	Simvastatin + Fluconazole
		Rodero et al. (2000)	AMB + 5FC, AMB + Rifampicin, FCZ + 5FC
		Venturini et al. (2011)	Amphotericin + Voriconazole
In vivo efficacy	Mice, rats: death curve, fungal burden, histopathology	Li et al. (2012)	Pogostone from *Pogostemon cablin*
		Han and Lee (2005)	Berberin + AMB
		Han (2007)	Grape seed extract + AMB
		Masoko et al. (2010)	Extracts of *Combretum* and *Terminalia* species
		Zhang et al. (2005)	Saponins from *Tribulus terries*
	G. mellonella: death curve, fungal burden, histology	Favre-Godal et al. (2014)	Anthraquinones from *Morinda tomentosa*
	C. elegans: death curve, fungal burden	Breger et al. (2007)	Different natural compounds
		Coleman et al. (2010)	Saponins
		Zhao et al. (2013)	Tetrandrine alkaloid
		Sun et al. (2015)	Neolignans of *M. officinalis*
In vitro toxicity	Colorimetric assays (MABA, MTT, XTT, sulforhodamine B)	Edziri et al. (2012)	*Retama raetam*
		Lee el al. (2012)	*Pleurospermum kamtschaticum*
		Lee et al. (2014)	*Rudbeckia laciniata*
		Mahlo et al. (2013)	*Breonadia salicina*
		Mokoka et al. (2013)	*Maytenus undata*
		Yousefzadi et al. (2009)	*Tanacetum balsamita*
		Zapata et al. (2010)	*Asteraceae* plant family
		Zarai et al. (2012)	*Ricinus communis*
In vivo toxicity	Mammals models- mice, rats	Fontenelle et al. (2007)	Essential oil from *Lippia sidoides*
		Regner et al. (2011)	Extract of *Pterocaulon polystachyum*
		Li et al. (2012)	Pogostone from *Pogostemon cablin*
		Castro et al. (2015)	Essential oil from *Psidium cattleianum*
	Zebrafish	Berry et al. (2004)	Pahayokolide A from *Lyngbya*
	G. mellonella	Gibreel and Upton (2013)	Epidermicin
	C. elegans	Pukkila-Worley et al. (2009)	Fluconazole
In silico Pharmacokinetics	Computational tool VolSurf	Djeddi et al. (2008)	Sesquiterpene lactone of *Centaurea pullata*
		Karioti et al. (2011)	Proanthocyanidins
In vitro Pharmacokinetics	Caco-2 cells	Takano et al. (1998)	Ketoconazole
	Microsomes and hepatocytes	Wind et al. (2009)	Piperazine propanol derivative
In vivo Pharmacokinetics	Mice, rats	Li et al. (2012)	Compound of *Pogostemon cablin*
New application	PDT	Gasparetto et al. (2010)	Extracts of *A. maritima*
	PDT	Rezusta et al. (2012)	Hypericin
	PDT	Paz-Cristobal et al. (2014)	Hypericin
	PDT *in vivo*	Dovigo et al. (2013)	Curcumin
	Nanotechnology	Bhawana et al. (2011)	Curcumin
	PDT and nanotechnology	Luo et al. (2009)	Allicin
		Svetlichny et al. (2015)	Copaiba oil
		Baltazar et al. (2015a)	Curcumin
		Chen et al. (2012)	Chitosan nanoparticules loaded with erythrosine
		Garg and Singh. (2011)	Eugenol nanoparticules

chitin, has been used in the manufacture of nanoparticles to improve drug delivery. The use of nanoparticles of chitosan loaded with erythrosine were active against *C. albicans* (planktonic and biofilm) after PDT application (Chen et al., 2012). Curcumin is a yellow-orange polyphenol compound produced by the rhizome of *Curcuma longa* plants with a wide range of pharmacological activities (Martins et al., 2009). Baltazar et al. (2015a) evaluated *in vitro* the activities and the mechanism of action of a photoactivated free and encapsulated curcumin against a clinical strain of *T. rubrum*. The results showed that curcumin aPI is an effective alternative for the treatment of *T. rubrum* infection, inducing the increase of nitric oxide (NO) and promoting apoptosis of fungal cells. According to many authors it still needs more studies to assert that nanotechnology may enhance photodynamic therapy. Despite the many reports in the literature of the use of both photodynamic therapy and nanotechnology for the treatment of cancer, there are very few studies on the use of both for antifungal treatment.

Table 1 provides examples of studies applying the methodologies discussed in this review.

5. Conclusion

Recently, the need for search and development of new compounds with antifungal activity has increased. The therapeutic failure, development of resistant strains, and low availability of antifungal drugs has led to natural compounds to be widely studied. Furthermore, choosing the correct assays as well as considering the compound and the fungi is important. A sequence of *in vitro* methods followed by *in vivo* models to assess the pharmacology and toxicity is generally used for compounds against fungi in a preclinical stage. To evaluate the antifungal activity of compounds, isolated or combined, macro- and micro-dilution, agar diffusion and colorimetric methods are available for the verification of cell viability. Cytotoxicity should also be performed, being essential before initiating animal testing. Then, the toxicology, efficacy, and pharmacokinetics studies should be employed, starting with alternative models and followed by mammalian models. Finally, photodynamic therapy and nanotechnology have been a great ally in the pursuit of new strategies to combat fungal infections.

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