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Análise proteômica do fungo *Paracoccidioides* durante o processo infeccioso em macrófagos

TESE DE DOUTORADO

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SHEYLA MARIA RONDON CAIXETA BONFIM

Análise proteômica do fungo *Paracoccidioides* durante o processo infeccioso em macrófagos

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Medicina Tropical e Saúde Pública da Universidade Federal de Goiás para obtenção do Título de **Doutor em Medicina Tropical e Saúde Pública.**

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"Hei de Vencer.
Hei de Vencer-me!
Pois, só aqueles que sejam
capazes de vencer suas próprias
fraquezas, limitações e erros,
é que alcançam a
verdadeira vitória."

Arthur Riedel

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SUMÁRIO

FIGURAS E TABELAS

SÍMBOLOS, SIGLAS E ABREVIATURAS

AMPc: Adenosina Monofosfato cíclico

APC: Célula apresentadora de antígeno

ATP: Adenosina Trifosfato

BHI: Meio de cultura infuso coração e cérebro

BMDM: Macrófagos derivados da medula óssea

CFSE: Éster Carboxi Fluoresceína Succinimidil

DNA: Ácido Desoxirribonucleico

EBP: Proteína de ligação ao estradiol

EDTA: Ácido Etileno Diamino Tetra Acético

ERG 11: citocromo p450 14 alfa esterol demetilase

EROS: Espécies reativas de oxigênio

GMCSF: Fator estimulador do crescimento de macrófagos

GPI: Glicosil fosfatidil inositol

GAPDH: Gliceraldeído fosfato desidrogenase

HSP: Proteína de choque térmico

IEF: Focalização isoelétrica

IFN- γ: Interferon gama

IPG-Phor: Equipamento usado para IEF

ITS: Espaçador interno transcrito

J 774 A1: Linhagem celular de macrófagos

Kb: Kilobases

KDa: Kilodalton

μg : micrograma

mg : miligrama

µL: microlitro

mL: mililitro

µM : micromolar

MALDI: Ionização a laser assistida por matriz

MCD: Metil citrato desidratase

MCL: Metil Citrato Liase

MCS: Metil Citrato Sintase

MS: Espectrometria de massas

NADH: Nicotinamida Adenina Dinucleotídeo reduzido

NADPH:Nicotinamida Adenina Dinucleotídeo Fosfato Reduzido

NANO UPLC MS ^{E}: Cromatografia líquida de alta eficiência acoplada à espectrometria de massas com método de aquisição alternativo

PRRs: Receptores de Reconhecimento Padrão

PAMPs: Padrões Moleculares Associados aos Patógenos

PBS: Tampão Fosfato Salino

*Pb*01, *Pb18* : isolados 01 e 18 de *Paracoccidioides*

PCM- Paracoccidioidiomicose

PCR: Reação em Cadeia da Polimerase

qRT-PCR: PCR em tempo real

pI: ponto isoelétrico

PGDF: Fator de crescimento derivado de plaquetas

PGF-ß: Fator de crescimento de plaquetas ß

PMF: Método de identificação das proteínas pelo padrão de digestão enzimática

PS2: Espécie filogenética 2

PS3: Espécie filogenética 3

PS4: Espécie filogenética 4

RNI- Intermediários reativos de nitrogênio

RPM: Rotações por minuto

S1: Espécie filogenética 1

SFB: Soro Fetal Bovino

TLRs: Receptores do tipo Toll

TNFα: Fator de necrose tumoral alfa

TOF: Tempo de voo

2D-PAGE: Eletroforese bidimensional em gel de poliacrilamida

RESUMO

O fungo *Paracoccidioides* é um patógeno humano causador da micose sistêmica paracoccidioidomicose (PCM). A infecção ocorre através da inalação de propágulos do fungo pelo hospedeiro e os macrófagos são importantes na contenção inicial do fungo por meio de mecanismos inatos. Neste estudo realizamos a análise do perfil proteômico de células leveduriformes de *Paracoccidioides,* isolado *Pb*18, recuperadas da infecção em macrófagos J774 A1 para identificar moléculas que são expressas nesta condição e poderiam representar alvos para novas terapias antifúngicas. Nas análises de expressão diferencial de proteínas foi possível detectar 181 proteínas com indução da expressão e a diminuição da expressão foi observada em 245 proteínas em *Pb*18. Os dados obtidos revelam a regulação positiva de proteínas nos processos envolvidos no metabolismo alternativo do carbono como gliconeogênese, beta-oxidação de ácidos graxos e catabolismo de aminoácidos. As proteínas com diminuição nos níveis de expressão incluem aquelas relacionadas à glicólise e síntese proteica. Ainda proteínas envolvidas na resposta ao estresse oxidativo e defesa celular apresentam aumento nos níveis de expressão tais como superóxido dismutase, proteínas de choque térmico, tioredoxinas e citocromo C peroxidase. Um mutante foi gerado para avaliar a importância da enzima citocromo c peroxidase durante a infecção sugerindo o envolvimento em um complexo sistema de proteção contra o estresse oxidativo, reforçando papel na sobrevivência do fungo. O perfil proteômico de *Paracoccididioides spp* em resposta à internalização em macrófagos, descrito pela primeira vez, reflete significativo remodelamento do metabolismo do fungo frente ao estresse oxidativo e ressalta a versatilidade do fungo em adaptar-se ao ambiente hostil do macrófago buscando novas estratégias de sobrevivência.

Palavras-chave: *Paracoccididioides,* macrófago, infecção, análises proteômicas

ABSTRACT

Paracoccidioides is a fungal human pathogen that causes systemic mycosis paracoccidioidomycosis (PCM). Infection occurs via inhalation of fungal propagules by the host and macrophages are important in the initial contention of the fungus through innate mechanisms. In this study, analysis of proteomic profile of yeast cells of *Paracoccidioides,* isolate *Pb*18, recovered from infection in macrophages J774 A1 so as to identify molecules that are expressed in this condition and could represent targets for new antifungal therapies. The analysis of differential protein expression could be detected 181 proteins with induction of expression and decreased expression was observed in 245 proteins in *Pb18*. The data show up regulation of proteins involved in the processes on the alternative carbon metabolism such as gluconeogenesis, beta-oxidation of fatty acids and amino acids catabolism. Proteins with decreased levels of expression include those related to protein synthesis and glycolysis. Aditionally proteins involved in oxidative stress response and cell defense exhibit increased levels of expression such as superoxide dismutase, heat shock proteins, cytochrome c peroxidase and thioredoxins. A mutant was generated to assess the importance of the enzyme cytochrome c peroxidase during infection suggesting involvement in a complex system of protection against oxidative stress, reinforcing role in the survival of the fungus. The proteomic profile of *Paracoccididioides sp* in response to internalization in macrophages, first described, reflects significant remodeling of fungal metabolism against oxidative stress and highlighting the versatility of the fungus to adapt to the hostile environment of the macrophage seeking new strategies survival.

Keywords: *Paracoccidioides*, macrophage, infection, proteomic analyses

1 INTRODUÇÃO / REVISÃO DA LITERATURA

1.1 Paracoccidioides : Aspectos Gerais

As micoses sistêmicas são infecções causadas por fungos patogênicos que podem ter como porta de entrada o trato respiratório e disseminar-se por todo o organismo. As micoses sistêmicas endêmicas no Brasil são: Paracoccidioidomicose, Histoplasmose, Coccidioidomicose, Criptococose (SUVISA 2012). A Paracoccidioidomicose (PCM) causada pelo fungo *Paracoccidioides* representa um importante problema de saúde pública, pelo seu potencial incapacitante quando não diagnosticada e tratada oportunamente (Shikanai-Yasuda *et al*., 2006).

O fungo *Paracoccidioides,* descrito por Adolpho Lutz em 1908, é o agente etiológico da Paracoccidiodomicose (PCM). O fungo foi isolado de lesões orais e de linfonodo cervical no Instituto Biológico de São Paulo-Brasil e é descrito como um parasita intracelular facultativo. Dados sugerem que ele seja um organismo diplóide, apresente o diâmetro que varia de 5 a 25 um (Desjardins *et al.,* 2011). Já foram finalizados os genomas estruturais de três isolados de *Paracoccidioides* (*Pb*01, *Pb*03 e *Pb*18) através do projeto denominado “Genômica Comparativa de *Coccidioides* e outros Fungos Dimórficos”. Para o isolado *Pb*01, o genoma é composto de 32,94 Mpb, com um total de 9.132 genes, sendo que apresenta o maior genoma tanto em número de bases quanto em quantidade de genes comparado aos outros dois isolados analisados, que apresentaram genomas do tamanho de 29,06 e 29,95 Mpb, com número de genes de 7.875 e 8.741 (dados dos isolados *Pb*03 e *Pb*18, respectivamente) (Broad Institute, http://www.broad.mit.edu/tools/data/seq.html).

O fungo, caracterizado por Almeida (1930), é classificado como pertencente ao reino Fungi, ao filo Ascomycota subdivisão Euascomycotina, à classe Plectomyceto, subclasse Euascomycetidae, à ordem Onygenales, à família Onygenacea e subfamília Onygenaceae Anamórficos, gênero *Paracoccidioides* (San-Blas *et al*., 2002). Considerando a análise taxonômica de *Paracoccidioides* realizada pelo uso de diferentes métodos moleculares, diferenças genômicas das linhagens mostram a evidência de um complexo que incluem quatro diferentes espécies filogenéticas: S1 (espécie 1), PS2 (espécie filogenética 2) e PS3 (espécie filogenética 3) e PS4 (espécie filogenética 4). A espécie filogenética S1 está distribuída no Brasil, Argentina, Paraguai, Peru e Venezuela, alguns isolados da espécie filogenética PS2 foram encontrados no Brasil nos estados de São Paulo e Minas Gerais e ainda na Venezuela; PS3 está geograficamente restrita à Colômbia (Matute *et al.,* 2006) e PS4 que foi recentemente descrita em isolados obtidos da Venezuela (Theodoro *et al*., 2012). O isolado *Pb*01, referido como "*Pb*01-like", apresentou diferenças genéticas em relação às outras linhagens (Carrero *et al.*, 2008), e pode ser sugerido como uma nova espécie filogenética. Alguns autores sugerem a mudança do nome para *Paracoccidioides lutzii*, em homenagem a Adolfo Lutz (Teixeira *et al*., 2009).

Em áreas endêmicas, foi identificada a presença de *Paracoccidioides* em animais silvestres como tatus (*Dasypus novemcinctus)* (Silva-Vergara *et al*., 1999) e *Cabassous centralis* na Colômbia, o macaco *Saimiri sciureus* (Johnson *et al*, 1977), morcego *Artibeus lituratus* (Grosssi *et al*., 1965), pingüim *Pygoscelis adeliae* (Gezuele *et al.*, 1989) entre outros; bem como em animais domésticos, como cães (*Canis lupus familiaris)* (Cantero *et al.*, 2010), gatos (*Felis domesticus*) e ovelhas (O*vis aries)* o que sugere que esses animais possam ser reservatório natural do fungo (Bagagli *et al*., 2003; de Farias *et al*., 2011; Gonzalez *et al.*, 2010; Oliveira *et al*., 2012; Richini-Pereira *et al*., 2008).

Apesar do fungo ter sido isolado do solo, do trato digestivo e das fezes de alguns animais, a caracterização do habitat natural do fungo ainda não está totalmente definida (Lacaz *et al*., 1999) e sua ecologia é praticamente desconhecida devido a dificuldades de isolamento ambiental, a localização deste patógeno no ambiente, o prolongado período de latência da doença e migrações das populações. Acredita-se que o fungo seja um organismo saprobiótico do solo (Albornoz, 1971; Silva-Vergara *et al*., 1998, Restrepo *et al*., 2001)

Uma importante característica de fungos ascomicetos, como *Coccidioides immitis*, *Sporothrix schenkii*, *Penicillium marneffei*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* e *Paracoccidioides*, é a habilidade de alterar sua morfologia de forma filamentosa multicelular miceliana para forma unicelular leveduriforme, caracterizando o dimorfismo celular (**Figura 1**).

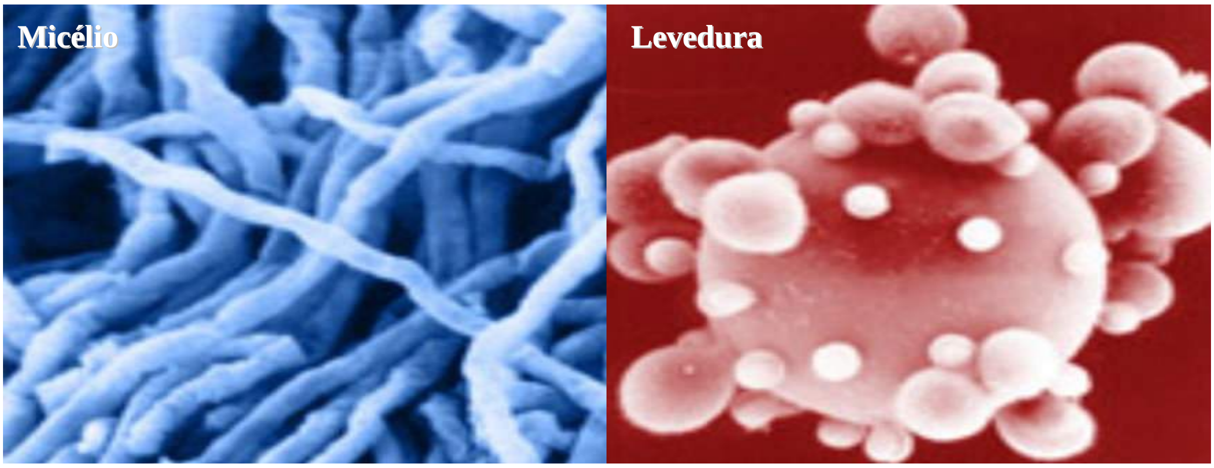

Figura 1: Aspectos morfológicos de *Paracoccidioides spp.* As fases miceliana (esquerda) e leveduriforme (direita) são mostradas em visão microscópica do fungo.
Fonte: http://www.broadinstitute.org/

O fungo *Paracoccidioides* é considerado um fungo dimórfico e apresenta duas formas: a miceliana e a leveduriforme. A forma miceliana apresenta crescimento em micélios aéreos curtos bastante aderidos ao meio de cultura com hifas septadas, presença de artrósporos e clamidoconídeos intercalares (Marques, 1998) em temperaturas que variam de 23ºC - 25ºC sob condições saprobióticas. Quando inalados pelo humano dão origem à formas semelhantes à leveduras, que caracterizam a forma parasitária, sob temperaturas que variam de 35ºC - 37ºC (San-Blas *et al*., 2002) como observadas em microscopia de campo claro e fluorescência (**Figura 2**).

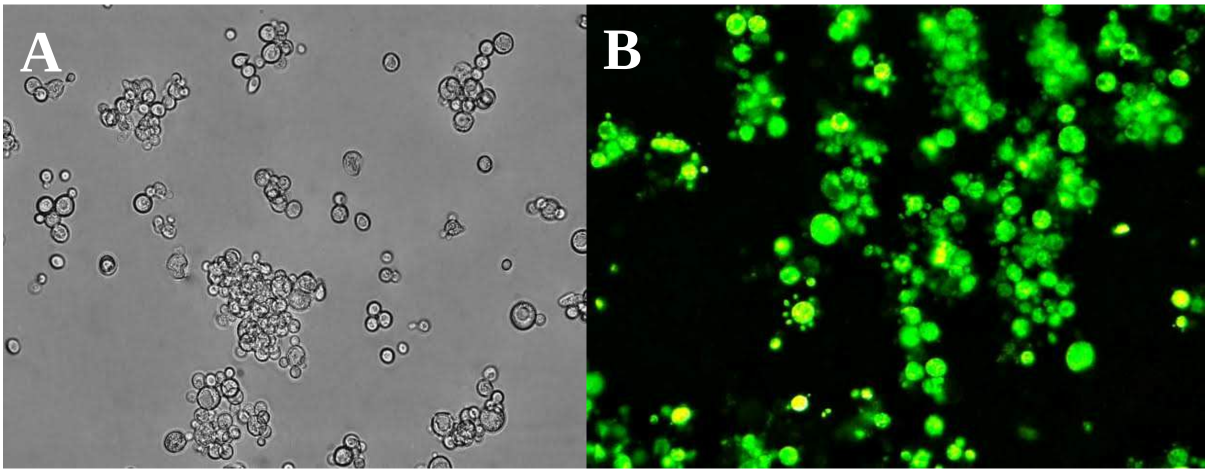

Figura 2 : Microscopia da fase leveduriforme do fungo *Paracoccidioides* isolado *Pb 01*. A forma leveduriforme do fungo *Paracoccidioides isolado Pb01* mostrada em microscopia campo claro com aumento de 400x (A) e em imunofluorescência com uso do fluoróforo CFSE- Carboxy Fluorescein Succinimidyl Ester (B)
Fonte: Laboratório de Biologia Molecular/UFG/ Sheyla Ma.R.C.Bonfim.

A transição morfológica pode ser influenciada por fatores como nutricionais e temperatura do ambiente, solos com alta umidade que devem ser favoráveis para a produção de conídios, fatores importantes por aumentar a eficiência de sobrevivência do fungo no meio ambiente e a capacidade de infectar animais e humanos (Terçarioli *et al.,* 2007).

A mudança morfológica do fungo é acompanhada por mudanças na parede celular. A parede celular de ambas as formas miceliana e leveduriforme do fungo *Paracoccidioide*s é responsável pela manutenção da forma celular e estabilidade, apresenta-se constituída principalmente por quitina, glucanas e proteínas. A parede de micélio tem uma grande concentração de proteínas (24 a 41%) quando comparada com células leveduriformes (7 a 14%) (Kanetsuna *et al*. 1969).

O principal polissacarídeo da parede celular de leveduras de *Paracoccidioides* é a α-glucana, enquanto que em micélio são as β-glucanas e as galactomananas (aproximadamente

6%). As α-glucanas de leveduras contêm pequenas concentrações de ligações glicosídicas α-1,3 ou α-1,6, sendo que as β-glucanas de micélio contêm ligações glicosídicas β-1,3. Em micélio, a parede celular apresenta uma única camada contendo quitina e β-glucana (Carbonell & Rodriguez 1968) enquanto que foram observadas três camadas em levedura em estudos de microscopia eletrônica; a camada interna é formada de β- glucana, a externa de α-glucana (Carbonell,1963) e a camada intermediária que ainda não apresenta constituição descrita. Considerando que moléculas como glucana e quitina estão ausentes no hospedeiro humano, elas podem representar potentes alvos de drogas antifúngicas que geralmente bloqueiam a síntese de glucana da parede celular sem interferir com outro processo metabólico no hospedeiro. A fase leveduriforme possui duas vezes mais lipídeos que a miceliana, fato explicado pela necessidade de manutenção da permeabilidade da membrana e sobrevivência em temperaturas de 36°C (Hamdan *et al*., 1993). Os glicoesfingolipídeos, lipídeos com função estrutural, estão envolvidos no direcionamento de proteínas que apresentam âncoras do tipo GPI (glicosil fosfatidil inositol) e que apresentam importante papel na remodelagem da parede celular (Kaur *et al*., 2007).

1.2 A Doença Paracoccidioidomicose (PCM)

A PCM, causada pelo fungo *Paracoccidioides* (Restrepo *et al*., 2003), representou a mais importante causa de mortes dentre as micoses sistêmicas avaliadas no período de 1996-2006 no Brasil com ocorrência de aproximadamente 51,2% dentre os casos avaliados (Prado *et al*.,2009). A distribuição geográfica está diretamente relacionada ao clima (Rivitti *et al*., 1999), sendo que a maioria dos casos ocorre em áreas tropicais, com solos ácidos e com temperaturas que variam de 17°C a 24°C. A PCM apresenta-se geograficamente restrita à América do Sul e América Central (Restrepo & Tobón, 2005, Marques *et al*., 2012)

A principal via de entrada do parasita é por inalação de propágulos do fungo pelo hospedeiro humano (Lacaz *et al*. 1991). No homem, micélios ou conídios convertem-se em leveduras, que reproduzem-se por brotamentos múltiplos ou simples nos tecidos infectados, sendo fagocitados por macrófagos (Brummer *et al*. 1988). Para o estabelecimento da PCM no hospedeiro, o processo de transição dimórfica é o primeiro requerimento (Lacaz *et al*. 1991).

Um dos aspectos peculiares da doença é a sua prevalência por sexo e idade. A incidência clínica e progressão de PCM em áreas endêmicas é maior em homens em relação à mulheres (Ramos *et al*., 2008), e em homens adultos trabalhadores de área rural (Silva-Vergara *et al*., 1998), a forma crônica é mais prevalente. Dados epidemiológicos reforçam a hipótese de que fatores hormonais tem papel crítico na patogênese da doença. Acredita-se que o hormônio 17 β-estradiol, um dos hormônios estrógenos femininos, possa oferecer proteção à mulheres adultas em idade fértil pela inibição da transição de micélio para levedura (Aristizabal *et al.*, 1998, Sano *et al.*, 1999) sendo uma evidência de que os hormônios podem afetar o padrão de virulência do fungo. No estudo de Pinzan *et al*, 2010, foi mostrado que a influência do sexo na PCM atribuída à ação hormonal pode ser observada pela presença de maior resistência à doença em camundongos fêmeas caracterizando um perfil diferenciado na resposta imunológica. Alta produção de citocinas Th1 em cultura de células de camundongos fêmeas infectadas estimuladas com paracoccina foi observada, e em células de camundongos macho infectadas a produção foi de citocinas Th2. Os resultados mostram que os hormônios sexuais tem profundo efeito na biologia de células do sistema imune e a atuação do estradiol na inibição da transição de micélio para leveduras favorece o efeito protetor frente à infecção. Acredita-se que a interação do hormônio com a proteína EBP (*Estradiol Binding Protein*) identificada em *Paracoccidioides*, iniba a transição morfológica do fungo, explicando a baixa incidência da PCM em mulheres (Shankar *et al.*, 2011).

A classificação das formas clínicas de PCM adotada (Medellín, Colômbia, 1986) é baseada na correlação de dados clínicos com a história natural da doença, conforme mostrada na Quadro 1.

Quadro 1. Classificação clínica da paracoccidioidomicose

1. Paracoccidioidomicose infecção
2. Paracoccidioidomicose doença
    - 2.1 Forma Aguda
        - 2.1.1 Moderada
        - 2.1.2 Grave
    - 2.2 Forma Crônica
        - 2.2.1 Unifocal
            - Leve
            - Moderada
            - Grave
        - 2.2.2 Multifocal
            - Leve
            - Moderada
            - Grave
3. Paracoccidioidomicose associada à imunossupressão
4. Forma residual (sequela)

A maioria dos indivíduos infectados desenvolve a forma assintomática ou subclínica, a qual progride para doença com uma diversidade de formas em função de fatores do hospedeiro, níveis de virulência das linhagens e condições ambientais (San-Blas *et al.*, 2002). A infecção primária ocorre pela inalação de propágulos do fungo pelo homem, iniciando nos pulmões e podendo disseminar-se para outros órgãos através dos linfonodos e da corrente sanguínea. As lesões secundárias podem ocorrer em membranas mucosas, pele, linfonodos e glândulas adrenais (Brummer *et al.*, 1993).

Três níveis de evolução podem ser observados durante a forma assintomática da PCM. O primeiro refere-se à regressão do complexo primário com a destruição total dos fungos; o segundo a regressão com a persistência dos fungos viáveis; e o terceiro a progressão para o desenvolvimento da doença (Franco *et al.*, 1989, Franco *et al.*, 1998). A evolução do complexo primário pode induzir à PCM doença e também à reativação dos focos latentes, proporcionada por um desequilíbrio entre o hospedeiro, parasita e ambiente (Marques, 1998).

A doença desenvolve-se em dois diferentes padrões: a forma sub-aguda ou aguda e a forma crônica. As formas disseminada sub-aguda e pulmonar aguda tem sido descritas como a forma juvenil, com um pico de incidência entre 10 e 20 anos de idade. Existe o envolvimento do sistema fagocítico e os sintomas apresentados são: febre, e manifestações como linfoadenomegalia e hepatoesplenomegalia (Benard *et al*., 2008) e aproximadamente 5-10 % dos casos ocorrem nesta fase. As formas disseminada e pulmonar crônica tem sido encontradas em adultos jovens (Franco, 1987) e os sintomas podem estar associados à um único órgão (unifocal) ou vários órgãos (multifocal) usualmente envolvendo os pulmões e mucosas do trato respiratório, orofaringe, pele, linfonodos e glândulas adrenais com descrição de insuficiência respiratória e Doença de Addison (Queiroz-Telles *et al.,* 2011). A forma crônica de PCM afeta homens com 30 a 50 anos de idade e representa um total de 90% dos casos descritos. O envolvimento pulmonar, usualmente acompanhado de lesões na mucosa oral e nasal, é o aspecto mais frequentemente encontrado na prática clínica. Essa forma apresenta curso lento e silencioso requerendo meses ou anos para ser diagnosticada (Morejón *et al*., 2009).

O diagnóstico de PCM deve ser baseado em dados clínicos, epidemiológicos e microbiológicos. Em procedimentos de rotina laboratorial, o diagnóstico para PCM é baseado na observação direta por visualização microscópica de elementos sugestivos de *Paracoccidioides* a partir de amostras obtidas de biópsia de pele, mucosa, linfonodos; material obtido de raspados de lesões mucocutâneas ou abcessos e aspirados de lesões ganglionar. A morfologia característica do fungo é representada por células grandes circundadas por múltiplos brotamentos com aspecto em "roda de leme". O exame histopatológico é realizado em áreas necróticas de infecção secundária que apresentam granuloma rico em células epitelióides e gigantes algumas contendo variadas concentrações de parasitas e aporte celular como linfócitos, plasmócitos, eosinófilos e áreas de exsudação contendo neutrófilos. A visualização microscópica é possível mediante coloração por Hematoxilina Eosina (HE), Grocott-Gomori, Azul de Toluidina (Travassos *et al*., 2008; Brummer *et al*., 1993) como mostrado na (**Figura 3**).

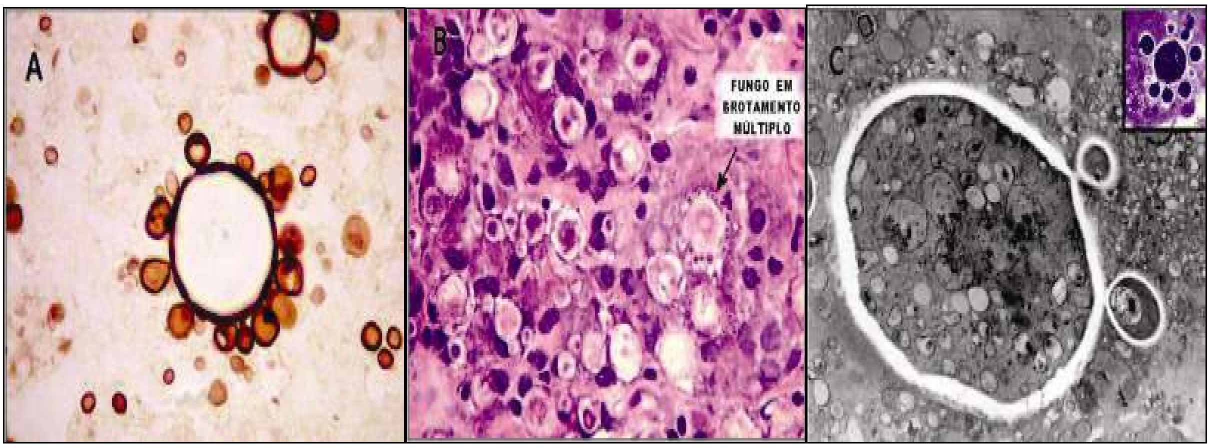

Figura 3: Imagens microscópicas do fungo *Paracoccidioides spp* observadas em *c*ortes de linfonodos com diferentes colorações. (A) Cortes incluídos em parafina corados por Grocott com impregnação argêntica para detalhes da morfologia do fungo como o brotamento múltiplo em roda de leme em 1000x, (B) coloração com Hematoxilina Eosina HE em 400 x, (C) coloração com Azul de toluidina em material incluído em Araldite e na inserção, um fungo corado com aspecto semelhante em microscopia eletrônica.
Fonte: http://anatpat.unicamp.br/biinflparacoco3.html/http://anatpat.unicamp.br/biinflparacoco1.html

Testes sorológicos específicos são úteis para detecção de anticorpos anti–*Paracoccidioides* quando as lesões não são facilmente acessíveis e para controle terapêutico no monitoramento da resposta do paciente ao tratamento. Testes como imunodifusão dupla, imunofluorescência indireta, ensaios imunoenzimáticos com detecção de antígenos gp43 e gp 70 (Marques da Silva *et al*., 2004) estão disponíveis. A presença de reações cruzadas com outras micoses (Albuquerque *et al.*, 2005), alta sensibilidade com baixa especificidade e alto custo podem ser fatores limitantes no uso desses ensaios no diagnóstico (Do Valle *et al*., 2001).

Métodos moleculares vêm sendo empregados para a detecção de *Paracoccidioides* através da PCR (Reação em Cadeia da Polimerase) avaliando regiões do DNA, que compreendem o ITS1 (*Internal Transcribed Spacer 1*), 5.8S e ITS2 (*Internal Transcribed Spacer 2*), específicas do fungo *Paracoccidioides* (Theodoro *et al.*, 2005) e também hibridização de ácidos nucleicos. Estes testes estão ainda em estágios iniciais de aplicação para rotina podendo contribuir grandemente para estudos epidemiológicos e, se associados à tecnologias de biosensoriamento e bionanotecnologia poderão implementar o diagnóstico por detecção biomolecular de PCM (Queiroz-Teles *et al.*, 2011).

A estratégia terapêutica para tratamento da PCM inclui escolha da droga antifúngica e a duração do tratamento representa um desafio por ser realizada em longos períodos e frequência de seqüelas tem sido relatadas. A severidade da doença, o local da lesão e contra indicação para o uso de determinada medicação tem sido os critérios observados na condução da terapia (Yasuda *et al.,* 2005). Os antifúngicos disponíveis para o tratamento de PCM incluem sulfonamidas, polienos, fluorocitosina, equinocandinas e derivados azólicos. As sulfonamidas podem estar associadas ao trimetropim sulfametoxazol inibindo a síntese de ácido fólico, causando depleção do folato intracelular que é essencial para o crescimento do fungo (Hong et al., 1995).

Como polienos, a anfotericina B e nistatina atuam ligando-se à esteróis de membrana, como o ergosterol em fungos, aumentando a permeabilidade e podendo gerar nefrotoxicidade (Wu *et al*., 2005) e morte celular (Beauvais & Latgé, 2001). Embora a anfotericina B seja o antifúngico mais utilizado no tratamento de micoses sistêmicas, seu uso clínico tem sido limitado devido ao alto nível de toxicidade (Wu *et al*., 2005). Antifúngicos como a 5-Fluorocitosina (Krcmery, 2005), que inibe a síntese de ácidos nucleicos do fungo, e as equinocandinas, que ligam-se à beta glucanas, podem ser utilizados em terapia combinada com anfotericina B potencializando sua ação em efeito sinérgico contra *Paracoccidioides.* O advento dos derivados azólicos revolucionou o tratamento de PCM pela facilidade de administração, boa tolerância e diminuição dos efeitos colaterais. Os azoles representados por fluconazol, itraconazol, cetoconazol, posaconazol e voriconazol atuam interagindo com a enzima citocromo p450 14 alfa esterol demetilase ERG 11 ligando-se ao sítio ativo da enzima, causando inibição e depleção de ergosterol (Kelly *et al.,* 1993).

Uma outra opção que tem sido avaliada é o uso de vacinas. Vacinas baseadas em DNA usando gene gp43 mostram a imunidade protetiva contra o fungo em camundongos BALB C (Pinto *et al*., 2000). O peptídeo P10, um derivado de gp 43, é capaz de proteger contra infecção em camundongos (Kono *et al*., 2009) induzindo a resposta imune por produção de interferon-gamma (IFN-γ) e interleucina IL-12 (Marques *et al*., 2004).

A presença de aglomerados de células epiteliais com áreas centrais de necrose e agregados de leucócitos polimorfonucleares caracteriza a PCM (De Brito *et al*., 1994). Os polimorfonucleares e macrófagos estão presentes nas lesões após alguns dias de infecção e podem sofrer ativação por citocinas, mediadores biológicos responsáveis pela comunicação das células do sistema imune (Brummer *et al*., 1991). As citocinas ativam os macrófagos a destruir as leveduras e removê-las do hospedeiro, efeito fungicida ativado mediante a produção de interferon-gamma (IFN- γ) (Calvi *et al*., 2003).

As citocinas são polipeptídeos produzidos por diversos tipos celulares e que atuam por mecanismo parácrino (células vizinhas) ou autócrino (nas próprias células produtoras), sendo formadas em cascata, ou seja, uma citocina estimula sua célula-alvo a produzir mais citocinas. Alguns termos podem ser utilizados como linfocinas, citocinas produzidas por linfócitos ativados; monocinas, citocinas produzidas por monócitos; interleucinas, citocinas produzidas por células hematopoiéticas que agem principalmente em leucócitos; fatores de crescimento, citocinas que estimulam a proliferação celular e quimiocinas, citocinas que atuam como agentes quimiotáxicos. Elas ligam-se à receptores específicos, ativam mensageiros intracelulares e influenciam a atividade e a diferenciação celular podendo aumentar ou atenuar a resposta inflamatória, citocinas pró-inflamatórias e anti-inflamatórias respectivamente. Dentre as citocinas consideradas como pró-inflamatórias, temos as interleucinas IL-1, IL-2, IL-6, IL-7 e Fator de Necrose Tumoral (TNF, *Tumoral Necrosis Factor*). As citocinas anti-inflamatórias são as interleucinas IL-4, IL-10, IL-13 e FTCβ (fator transformador de crescimento β) (Sommer *et al.*, 2010; Curfs *et al.*, 1997). É importante considerar que é fundamental o equilíbrio entre os sinais pró-inflamatórios e anti-inflamatórios nas interações entre o hospedeiro e o fungo.

A principal defesa do hospedeiro contra o fungo *Paracoccidioides* é a resposta imune mediada por células. A resposta imune celular representa um mecanismo de defesa e os macrófagos tem papel importante na imunidade inata (Linehan *et al.*, 2000, Janeway, 2001). Os macrófagos representam uma das células de defesa contra o fungo e os polimorfonucleares PMNs apresentam também um efeito fungistático sobre leveduras do fungo que podem ser aumentados pela ação de interferon-gamma (IFN- γ) e também do fator estimulador do crescimento de macrófagos GMCSF (Kurita *et al.*, 2000, Fleetwood *et al.*, 2009). Os macrófagos liberam citocinas pró-inflamatórias como TNF que induz a expressão de moléculas de adesão essenciais para a patogênese de doenças inflamatórias (Zhang *et al.*, 2011).

Após a infecção, ocorrem as respostas inata e adaptativa e a progressão da doença varia em função da virulência da linhagem e estado imune do hospedeiro. Monócitos, macrófagos e neutrófilos fagocitam patógenos e estimulam a resposta de citocinas resultando no desenvolvimento da imunidade inata ou natural, a resposta inflamatória e imunidade adaptativa (Calich *et al.*, 2008).

O controle imunológico da PCM depende do padrão de resposta imune celular Th-1 com atividade pró-inflamatória, caracterizado pela produção de interleucina IL-2 e interferon-gamma (IFN- γ) que ativa macrófagos e linfócitos resultando na formação de granulomas. A produção aumentada de citocinas Th-2 com atividade anti-inflamatória apresenta envolvimento de interleucinas IL-4, IL-5, IL-6 e IL-10 que estimulam a resposta de células B com diminuição da produção de interferon-gamma (IFN- γ), padrões considerados típicos de pacientes que apresentam a forma severa da doença (Benard *et al.*, 2008). Estudos tem mostrado que indivíduos saudáveis que foram sensibilizados com *Paracoccidioides* produzem níveis substanciais de interferon gama, interleucinas IL-12 e IL-10 e pacientes que apresentam PCM produzem baixos níveis de IL-12, interferon gama, fator de necrose tumoral-TNF e níveis estáveis de IL-10 (Bava *et al.*, 1999). Este balanço na secreção de citocinas pode regular a resposta do hospedeiro por diminuir a atividade microbicida.

Os pacientes que desenvolvem a doença apresentam depressão da resposta Th-1 e predomínio da resposta Th-2 com ativação de linfócitos B e altos níveis de anticorpos anti-*Paracoccidioides* observados em testes sorológicos. O aumento no nível de anticorpo anti-gp43, tem sido demonstrado tanto em seres humanos quanto na PCM experimental utilizando camundongos susceptíveis como o B.10A e o Swiss (Mendes-Giannini *et al.* 1990; Vaz *et al.*, 1992; Miura, 2001; Ramos *et al*., 2005).

Como reforçado por Brummer *et al.*, (1989), a morte de *Paracoccidioides* é aumentada pela ativação de macrófagos evidenciando o papel das citocinas na resistência do hospedeiro ao fungo. Considerando este contexto, macrófagos peritoneais e pulmonares que foram ativados por interferon-gamma (IFN- γ) matam os conídios do fungo como mostrado em estudos de microscopia que confirmaram a morte e digestão de *Paracoccidioides* em 48 horas, enquanto que macrófagos peritoneais ativados com Fator de Necrose Tumoral (TNF, *Tumoral Necrosis Factor*) inibem a transição de conídios para leveduras.

1.3 A Interação Patógeno e Hospedeiro

O estudo do mecanismo de interação do patógeno e hospedeiro nas doenças infecciosas pode ajudar a conhecer mais sobre a biologia de *Paracoccidioides* e desenvolver novos alvos para terapias e vacinas (Fortes *et al.*, 2011).

O contato do fungo com o tecido do hospedeiro desencadeia inicialmente reação inflamatória. A inflamação é um processo complexo iniciado por dano tissular que apresenta sinais característicos como edema, rubor, calor, dor e perda da função da área inflamada. A resposta inflamatória está diretamente ligada ao processo de reparação tecidual que inicia-se durante a fase inicial da resposta inflamatória (Robbins *et al.*, 2000). Como resposta fisiológica, fatores como migração e adesão leucocitária, vasodilatação, quimiotaxia de células como neutrófilos e macrófagos, liberação de mediadores inflamatórios como os eicosanóides derivados dos ácidos graxos essenciais, e liberação de fatores de crescimento e agregação plaquetária podem ser observados (Douglas *et al.*,2000). Os macrófagos secretam fatores de crescimento capazes de promover a proliferação vascular e fibroblástica, que auxiliam no reparo dos tecidos danificados durante o processo inflamatório (Delavary *et al.*, 2011). A liberação de produtos inflamatórios pelo sistema complemento e a ativação de plaquetas e células endoteliais podem caracterizar a fase proliferativa e contribuem para o recrutamento de fibroblastos para o local da lesão (Trowbridge & Emmling,2008). A liberação de fatores de crescimento como PGDF (Platelet Derived Growth Factor) e PGF-ß (Platelet Growth Factor ß) pode ocorrer devido à propriedade de quimiotaxia de macrófagos facilitando o reparo e cicatrização da lesão juntamente com ação de fibroblastos (Kumar *et al.*, 2005).

O processo crônico inflamatório granulomatoso pode ser dividido em três fases distintas: fase monocítica-neutrofílica caracterizada pela reunião concêntrica de macrófagos e polimorfonucleares no foco inflamatório induzindo à secreção de citocinas inflamatórias e produção do Fator de Necrose Tumoral (TNF) (Figueiredo *et al.*, 1993); fase pré-granulomatosa que ocorre com mobilização de macrófago e formação de células gigantes caracterizando granuloma do tipo epitelióide; fase granulomatosa com presença de infiltrado de células T ao redor do granuloma (Franco *et al.*, 1993).

A PCM é uma doença caracterizada pelo desenvolvimento de lesões granulomatosas com evolução crônica envolvendo órgãos e tecidos (Franco, 1987) em particular a pele, pulmões e mucosas oral, nasal e gastrintestinal. A PCM apresenta lesões com áreas extensas de necrose e processo inflamatório crônico com células gigantes, apresentando no interior delas fungos com a morfologia clássica de leveduras de *Paracoccidioides* e também infiltrado celular rico em linfócitos e plasmócitos. O granuloma na PCM representa uma resposta tecidual do hospedeiro contra o fungo na tentativa de destruir e impedir a multiplicação e disseminação (De Brito *et al.*, 1994).

A maioria das células envolvidas na resposta inflamatória é constituída de células fagocíticas que acumulam-se em torno da lesão, fagocitam o patógeno e liberam suas enzimas lisossomais. As células fagocíticas mononucleares compreendem monócitos, macrófagos e células dendríticas, sendo os monócitos os precursores dos macrófagos maduros e células dendríticas. Os monócitos representam 5% a 10% dos leucócitos periféricos e circulam no sangue por aproximadamente 24 horas, em seguida estes migram para os tecidos onde diferenciam-se em macrófagos (Abbas *et al.*, 2008).

O macrófago, descrito por Metchnikoff em 1884, apresenta diâmetro entre 25 a 50 um, superfície com grande número de projeções, pseudópodos e invaginações e citoplasma grande com presença de vesículas e vacúolos de secreção ou reserva (Santos & De Souza, 1983). Filamentos de actina e microtúbulos estão envolvidos na mobilidade dos pseudópodos (De Souza & Benchimol, 1985). Microscopicamente, o macrófago possui um núcleo regular com formato riniforme, a cromatina encontra-se dispersa, os lisossomos estão espalhados pelo citoplasma, as regiões do Golgi e Retículo endoplasmático rugoso também são desenvolvidas (Ian, 1973) e as mitocôndrias encontram-se em grande número (De Carvalho & De Souza, 1989).

Os macrófagos podem exibir morfologias diferentes e fundir-se formando células gigantes multinucleadas. Em função da localização, eles são conhecidos por várias nomenclaturas: macrófagos do tecido conjuntivo e linfóide, macrófagos alveolares no pulmão, células de Kupffer no fígado, microgliócitos no cérebro e macrófagos fusionados que produzem células gigantes multinucleadas como os osteoclastos (Gordon *et al*., 2005).

Durante o processo de interação patógeno–hospedeiro, os macrófagos apresentam grande importância durante a fagocitose, uma vez que atuam contra patógenos por meio da liberação de enzimas, geração de radicais livres, mediadores do processo inflamatório, apresentação de antígenos às células T funcionando como células apresentadoras APC- *Antigen Presented Cells* (Mc Greal *et al*., 2005). Em adição, os macrófagos apresentam função de remoção do material extracelular incluindo células apoptóticas, debris celulares e produtos metabólicos tóxicos (Mosser & Edwards, 2008).

Diferentes estados funcionais podem ser observados nos macrófagos como residentes, ativados e não ativados também chamados de quiescentes. Os macrófagos que são obtidos de organismos normais, ou seja, não infectados e sem inflamação são denominados residentes, apresentando capacidade mínima de destruir micro-organismos, secretando proteases em baixa quantidade, e pouca capacidade para responder a citocinas (Cohn *et al*., 1978).

A ativação de macrófagos pode ser definida como sendo a aquisição da capacidade de realizar funções complexas (Adans & Hamilton, 1984), funções essas que o macrófago residente não realiza. Eles podem ser ativados por via clássica ou por via alternativa via interleucinas IL-4 e Il-13. A ativação clássica de macrófagos leva a secreção de óxido nítrico e citocinas pró-inflamatórias, além de estimular a fagocitose. A ativação alternativa de macrófagos leva a secreção de citocinas anti-inflamatórias (Gordon *et al.,* 2003) como descrito no processo de infecção por *A.fumigatus* em que a defesa contra o fungo realizada por macrófagos alveolares revela a predominância de fenótipo de ativação alternativa (Bhatia *et al.*, 2011). Sabe-se que a ativação dos macrófagos está relacionada à resposta imunológica inata ou específica na qual o patógeno é apresentado a linfócitos T que quando ativados estimulam a liberação de interferon-gama (IFN- γ) (Muñoz-Fernandes *et al*., 1992), iniciando a ação efetora do macrófago pela produção de Fator de Necrose Tumoral (TNF) e óxido nítrico (Robbins *et al*., 1996; Adans & Hamilton, 1984). O espraiamento dos macrófagos representa um estado de ativação nos quais as células emitem pseudópodos e pode então contatar um agente estranho e a subsequente fagocitose. Este fenômeno pode ser observado na **Figura 4** pela presença de macrófagos obtidos de diferenciação a partir de células da medula óssea (BMDM) e macrófagos J774 A1 com morfologia esférica (residentes) e alongadas (espraiadas).

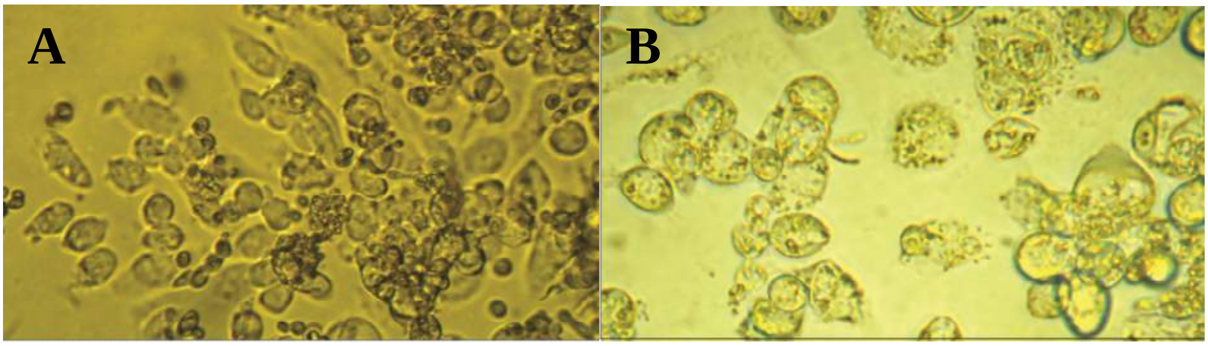

Figura 4: Os macrófagos em cultura celular. Imagens de microscopia óptica mostrando macrófagos em cultura celular em meio RPMI com aumento de 400X com morfologia esférica e alongada (espraiamento). A) Macrófagos BMDM obtidos por diferenciação de células da medula óssea de fêmur de camundongos na presença de GM-CSF 100 ng/ml e 5% CO2 (B) Macrófagos J774 A1 derivados de sarcoma reticular de camundongos.
Fonte: Laboratório de Biologia Molecular/UFG/ SheylaM.R.C.Bonfim)

Os macrófagos, ativados por interferon gamma e/ou lipolissacarídeos LPS, produzem dois tipos de produtos reativos como sendo os intermediários reativos de oxigênio e intermediários reativos de nitrogênio. O óxido nítrico e o peroxinitrito são os mais importantes intermediários reativos de nitrogênio produzidos por macrófagos e podem provocar a morte de patógenos como *C. neoformans* (Granger *et al*., 1988) e *C. albicans* (Diez-Orejas *et al*., 2001). Estudos "*in vitro*" sugerem que o fungo *Paracoccidioides* multiplica-se intracelularmente em macrófagos peritoneais não ativados e podem ser também destruídos em macrófagos ativados (Brummer *et al*.,1989).

A heterogeneidade observada nas diferentes populações de macrófagos é um fator importante, pois reflete a plasticidade destas células em resposta à exposição ao microambiente (Mantovani *et al*., 2004) como pela produção de citocinas. Considerando o perfil de ativação por citocinas, os macrófagos apresentam-se divididos em duas categorias: M1 e M2. Os macrófagos M1 induzidos por interferon-gama (IFN- γ) ou citocinas (Fator de Necrose Tumoral (*Tumoral Necrosis Factor*) e GMCSF (*Granulocyte Macrophage Colony-*

Stimulating Factor) expressam óxido nítrico sintase NOS2 e intermediários de oxigênio e nitrogênio, eficientes indutores de moléculas efetoras (ROS) (Gordon *et al*., 2003). Os macrófagosM2 induzidos por IL-4 e IL-13 expressam arginase (Martinez *et al.*, 2009) e apresentam capacidade variável de produção de citocinas inflamatórias.

Os macrófagos representam uma população de células que podem ser ativadas e exercer funções como reconhecimento e fagocitose mediadas por receptores (Greaves & Gordon, 2009). A fagocitose é um processo que caracteriza-se por apresentar internalização, morte e remoção de patógenos e partículas e ocorre em três etapas: o reconhecimento inicial, ingestão e degradação no interior de fagolisossomos. Uma vez que o patógeno é reconhecido, a fagocitose é iniciada e os mecanismos pró-inflamatórios celulares são ativados (Taylor *et al*., 2007). Após o reconhecimento do patógeno, os fagócitos iniciam o engolfamento levando à formação do fagossomo. Uma vez no fagossomo, o patógeno entra em contato com o meio ambiente microbicida do macrófago associado com a redução dos níveis de pH, presença de enzimas hidrolíticas, e também peptídeos antimicrobianos, geração de compostos tóxicos e enzimas lisossomais hidrolases, apresentando severa restrição e limitação de nutrientes. Após essa formação, o fagossomo pode fundir-se à endossomos e lisosssomos em um processo chamado de biogênese e maturação do fagolissomo. A maturação fagossomal é um processo gradual que envolve fusão de vesículas secretoras denominadas lisossomas, de neutrófilos e grânulos com o fagossoma, dando origem ao fagolisossomo. O mecanismo de destruição de micro-organismos no interior dos fagolisossomos é dependente de oxigênio: a fagocitose estimula o consumo de oxigênio e a produção de metabólitos reativos, que são convertidos em peróxido de hidrogênio (Desjardins *et al*., 1995).

O mecanismo de fagocitose de *Paracoccidioides* representa um processo mediado por receptores de manose (Jiménez *et al*., 2006). O receptor para manose é uma lectina, substância capaz de ligar-se à carboidratos e que pode atuar como sítio de reconhecimento celular, presente no macrófago que interage com resíduos terminais de manose e fucose de glicoproteínas e glicolipídeos no fungo. O reconhecimento por macrófagos mediado por receptores de manose também é observado em *Candida albicans* (Netea *et al.*, 2006).

O processo fagocítico deve ser considerado em quatro estágios distintos: acúmulo de fagócitos no local onde as células fúngicas estão localizadas, reconhecimento de PAMPs (*Pathogen Associated Molecular Patterns*) através de PRRs (*Pattern Recogniton Receptors*), engolfamento de patógenos que estão ligados à membrana do fagócito, processamento de células engolfadas dentro dos fagócitos pela fusão de vesículas lisossomais para formar o fagolisossomo (Kaposzta *et al*., 1999). A cinética de fagocitose é influenciada por dois fatores: forma e tamanho da partícula e interação com receptores específicos como PAMPs e PRRs (Hofman *et al*., 2010).

Os macrófagos quando estão em contato com o patógeno, são ativados e liberam moléculas antimicrobianas tóxicas como espécies reativas de oxigênio (EROS) como os intermediários reativos de nitrogênio (RNI- Reactive Nitrogen Intermediates) gerados pela iNOS (Inducible Nitric Oxid Synthase) gerados pelo sistema de NADPH oxidase nos fagócitos (Bocca *et al*., 1998;Youssef *et al*., 2012). O óxido nítrico é um dos produtos gerados por RNI e apresenta-se como um importante mediador inflamatório em resposta à infecção, é produzido a partir de L-arginina por atuação da enzima óxido nítrico sintase iNOS. Estudos em PCM indicam que o óxido nítrico parece exercer efeito modulador negativo na formação de granulomas e positivo durante a disseminação fúngica, o que favorece o desenvolvimento da doença (Nishikaku *et al.,* 2009).

Considerando que a PCM tem sido a maior causa de mortalidade dentre as micoses sistêmicas, o fungo *Paracoccidioides spp* representa um patógeno humano de grande relevância clínica. Fatores de virulência expressos pelo fungo durante o processo infeccioso tais como adesinas, mudança fenotípica, comportamento dimórfico, parede celular, melanina, secreção de enzimas hidrolíticas, podem contribuir para a persistência da colonização **(**Casadevall, 2007).

Avaliando o processo de adesão, a glicoproteína gp 43 de *Paracoccidioides,* antígeno secretado pelo fungo, é tida como fator de virulência devido à sua propriedade de adesina (Vicentini *et al*., 1994; Morais *et al.*, 2000), pela adesão do fungo à laminina e à matriz celular. A adesão do fungo à células epiteliais do hospedeiro é reduzida na presença de anticorpos anti gp-43 indicando que gp 43 atua como adesina (Mendes-Gianninni *et al.*, 2000). A expressão da proteína gliceraldeído fosfato desidrogenase (GAPDH), que localiza-se na camada mais externa da parede celular, foi avaliada e mostrou-se importante no processo de adesão por interagir com proteínas da matriz extracelular (Barbosa *et al*., 2006).

Para que ocorra uma colonização com sucesso, é necessário o envolvimento de proteínas na superfície do fungo, componentes de matriz extracelular e receptores celulares do hospedeiro (Sohn *et al*, 2006). Proteínas do hospedeiro como laminina, colágeno, fibronectina e fibrinogênio, podem ser ligantes celulares para os patógenos (Hanna *et al*, 2000). A habilidade da proteína enolase ligar-se à componentes da matriz extracelular e fibronectina sugere a atuação no processo de adesão (Marcos *et al*., 2012) podendo aderir à superfície de macrófagos J774 A.1 (Bailão *et al.*, 2012) além da atuação na via glicolítica. Também a paracoccina produzida pelo fungo, é capaz interagir com a laminina que favorece a adesão de leveduras e invasão de tecidos do hospedeiro importantes na patogênese da PCM (Coltri *et al.*, 2006).

Considerando a análise dos fatores de virulência, estudos tem mostrado a importância da melanina avaliando o impacto da sua produção durante a infecção, defesa e resistência à drogas antimicrobianas (Taborda *et al.*,2008). Melaninas são polímeros multifuncionais formados a partir da oxidação de compostos fenólicos usualmente apresentando coloração escurecida e são produzidas por uma variedade de micro-organismos patogênicos como bactérias e fungos. A presença de melanina em fungos é considerada um importante fator de virulência (Taborda *et al.*, 2008) sendo descrita em diversas espécies e presentes em fungos como *Paracoccidioides* e *Cryptococcus neoformans*. Estudos avaliam os mecanismos pelo qual a melanina é capaz de proteger células fúngicas do sistema imune do hospedeiro em *C.neoformans* (Nosanchuk *et al.*, 2000) *Sporothrix schenkii* (Romero-Martinez *et al.*, 2000) e *Paracoccidioides* (Gomez *et al.*,2001) atuando na proteção da ação de espécies reativas de oxigênio (EROS). Após a internalização do patógeno, respostas secundárias são ativadas como a produção de óxido nítrico e espécies reativas de oxigênio (EROS) e células melanizadas são mais resistentes à espécies reativas de oxigênio e também ao peróxido (Gonzalez *et al.*,2007). A melanina quando depositada na parede celular leva ao enrijecimento conferindo resistência celular, o que pode reduzir a fagocitose e também conferir proteção contra radicais oxidativos (Langfelder *et al.*, 2003).

Os fatores de virulência podem estar relacionados com os componentes estruturais do fungo. Alterações observadas na composição da parede celular durante a infecção apresentada pelo fungo *Paracoccidioides* reflete a transição de beta glucana para alfa glucana (Borges-Walmsley*et al.*, 2002). Beta glucanas são polímeros que estão presentes na parede celular de fungos, em plantas e bactérias que apresentam atividade imuno modulatória. Como os animais não apresentam este polissacarídeo, eles são considerados PAMPs clássicos reconhecidos pelo sistema imune inato de vertebrados via receptores celulares de superfície. O receptor Dectina-1 reconhece carboidratos que contém beta 1,3 e beta 1,6 glucana e é expresso nas células de monócitos, macrófagos e neutrófilos e em baixos níveis em células dendríticas (Taylor *et al.*, 2002). A quitina é um glicopolímero abundante que serve como componente estrutural de fungos mas não é sintetizado por vertebrados e pode induzir macrófagos a produzir citocinas pró-inflamatórias. A quitina induz à liberação de interferon-gama (IFN- γ), ativação de macrófagos alveolares (Shibata *et al.*, 2000) e expressão de arginase em macrófagos ativados alternativamente (Reese *et al.*, 2007). A quitina modula a expressão de ligantes nos macrófagos e portanto a eficiência da ativação de células T, diferenciação e proliferação (Wagner *et al.*, 2010).

Alguns fungos tem desenvolvido estratégias de sobrevivência dentro dos fagócitos por alterar o processo de maturação do fagolisossomo, promover detoxificação de compostos tóxicos, escape do fagolisossomo, adaptação ao pH e enzimas microbicidas. A análise da maturação do fagossomo pode ser útil para avaliar os mecanismos usados pelo fungo para sobreviver no meio intracelular de macrófagos. Em fagossomos maduros, a membrana forma um gradiente de prótons ATP-ase resultando em um decréscimo no pH, que favorece a atuação de enzimas lisossomais e morte do patógeno (Steinberg *et al.,* 2007).

Os fungos *Aspergillus fumigatus* e *Aspergillus terreus* exibem características distintas durante a interação com os macrófagos. O desenvolvimento da doença por *A. terreus* apresenta progressão mais lenta que *A. fumigatus* e persiste em estado de latência mas com viabilidade, resultando em baixa citotoxicidade em relação à *A. fumigatus* (Slesiona *et al.*, 2012).O fungo *A. fumigatus* inibe a acidificação do fagolisossomo e consegue sobreviver dentro do macrófago em um mecanismo de escape (Jahn *et al*., 2002, Ibrahim Graner *et al*., 2003).

Estudos descrevem que *C. glabrata* multiplica-se intracelularmente após ser fagocitada por macrófagos por modificar o meio do fagossomo para meio não acidificado sugerindo mecanismo de persistência e possível estratégia de evasão imune (Seider *et al.* 2010, Seider *et al*., 2014).

Ainda estudos mostrados por Eissenberg *et al.*, (1993) ressaltam que leveduras do fungo *Histoplasma capsulatum* sobrevivem dentro de fagossomos por regular o meio intracelular em macrófagos humanos e também em murinos pela diminuição da fusão ao fagolisossomo.

O estudo da interação entre o patógeno e o hospedeiro é importante na compreensão de aspectos relacionados à doença favorecendo o entendimento clínico e a elaboração de estratégias terapêuticas (Brakhage *et al*., 2010). Visando à identificação de proteínas e atividades enzimáticas envolvidas no processo de infecção, utilizamos como estratégia experimental a proteômica (Schmidt *et al*., 2011, Bumann,2010) no intuito de elucidar sobre a resposta do fungo *Paracoccidioides* em condições que mimetizam o processo infeccioso.

2 JUSTIFICATIVA

A infecção no hospedeiro humano causada pelo fungo *Paracoccidioides spp* ocorre com transição para a forma leveduriforme, caracterizada pela síntese de moléculas que interagem com várias células do sistema imune do hospedeiro e contribuem para a patogênese da doença. Um importante mecanismo na resposta imune contra vários patógenos é o processo de fagocitose onde os macrófagos tem um papel fundamental na resistência do hospedeiro ao fungo *Paracoccidioides* (Nascimento *et al*., 2002).

A análise da resposta de *Paracoccidioides* durante o processo infeccioso em macrófagos infectados através de estudos proteômicos, bem como análises comparativas de *Paracoccidioides spp* utilizando macrófagos da linhagem J774A1, pode fornecer informações sobre o envolvimento do fungo em processos celulares elucidando sobre alterações necessárias para sua sobrevivência e adaptação ao ambiente hostil do hospedeiro.

Ainda pouco é conhecido sobre a biologia do fungo na interação com o hospedeiro. Avaliando a capacidade do fungo sobreviver e multiplicar dentro de macrófagos, poderemos saber mais sobre mecanismos de latência e disseminação de *Paracoccidioides spp* no hospedeiro. A identificação de proteínas através da análise diferencial colabora no estudo da patogênese de PCM, e o conhecimento de moléculas envolvidas em condições de infecção podem ajudar no melhor entendimento do processo de interação entre o patógeno e o hospedeiro.

3 OBJETIVOS

3.1 Geral

Caracterização do perfil proteômico da forma parasitária de *Paracoccidioides Pb*18 recuperados de macrófagos infectados, mimetizando as condições de infecção no hospedeiro.

3.2 Específicos

1. Identificar proteínas diferencialmente expressas em leveduras recuperadas de infecção em macrófagos das linhagens J774 A1 nas análises proteômicas;
2. Caracterizar e classificar funcionalmente em categorias as proteínas obtidas;
3. Avaliar a resposta do fungo *Pb*18 no estudo das alterações metabólicas em condições de infecção em macrófagos;
4. Avaliar a eficiência do processo de fagocitose;
5. Realizar experimentos de confirmação da expressão diferencial de proteínas identificadas
6. Sugerir prováveis estratégias utilizadas pelo fungo visando à sobrevivência no meio hostil do hospedeiro;

4 MANUSCRITO

Paracoccidioides brasiliensis response to macrophage internalization

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ABSTRACT

Macrophages are key players during *Paracoccidioides brasiliensis* infection. However, the relative contribution of the fungal response to counteract macrophage activity remains poorly understood. In this work, we evaluated the *P. brasiliensis* proteomic response to macrophage internalization. A total of 426 proteins differentially expressed were detected in *P. brasiliensis* during macrophage infection. Among the positively regulated proteins we detected those involved in alternative carbon metabolism, such as enzymes gluconeogenesis, beta oxidation of fatty acids and amino acids catabolism. The down-regulated proteins during *P. brasiliensis* internalization in macrophages include those related to glycolysis and protein synthesis. Proteins involved in oxidative stress response are also up-regulated during macrophage infection in *P. brasiliensis* yeast cells, such as superoxide dismutases (SOD), thioredoxins (THX) and cytochrome c peroxidase (CCP). We generated an antisense knockdown mutant to evaluate the importance of CCP during macrophage infection.The result suggest that CCP is involved in a complex system of protection against oxidative stress and that the decrease in gene expression of this component of the antioxidant system diminishes the survival of *P. brasiliensis* inside macrophages.

INTRODUCTION

Paracoccidioidomycosis (PCM) is a human systemic mycosis restricted to Latin America, particularly Brazil, Colombia and Venezuela (San Blas *et al.*, 2002). The disease is caused by members of the *Paracoccidioides* genus, comprising, up to now, four cryptic species (Matute *et al.*, 2006; Matute *et al.*, 2006; Theodoro *et al.*, 2012; Teixeira *et al.*, 2009). These species are thermo-dimorphic, growing as mycelium under saprobic conditions at 22-26^0 C or as the pathogenic yeast at 36 ^{0} C (Brummer *et al.*, 1993). The saprobic form lives in soil. Upon the inhalation of spores or mycelial fragments by the host, these cells reach the lung alveoli, were they interact with epithelial cells and alveolar macrophages (McEwen *et al.*, 1987). At the host temperature, the fungus converts to the yeast pathogenic form (Lacaz 1994).

Macrophages constitute one of the primary defense mechanisms of the body against the infection by *P. brasiliensis* and PCM is considered a classic granulomatous disease (Brummer *et al.*, 1988; Brummer *et al.*, 1988). As a facultative intracellular pathogen, *P. brasiliensis* can persist inside macrophages, as the result of the defense mechanisms that allows the fungus to survive to the nutritional deprivation and the presence of oxidants (ROS) (Brummer *et al.* 1988; Brummer *et al.* 1988; Moscardi-Bacchi *et al.,* 1994). A notable characteristic of macrophages is the production of copious amounts of oxidants in the process of respiratory burst (Fang 2004).

During adaptation to the host, *P. brasiliensis* has been presumed to evolve the ability to respond to host-generated stress. In this sense, member of the *Paracoccidioides* spp. complex has been shown to express a powerful antioxidant defense system in the presence ROS-mediated oxidative stress. We have shown, by proteomic analysis, that the fungus presented a global activation of antioxidant enzymes, such as catalases, superoxide dismutases, cytochrome C peroxidase and thioredoxin when faced to H_2O_2. There was a shift in the metabolism of yeast cells, as suggested by the activation of the pentose phosphate pathway, a great source of cellular reducing power in the form of NADPH (de Arruda Grossklaus *et al.,* 2013). We have also shown that carbon starvation exert a strong effect on *Paracoccidioides* spp.. The stress, presumably similar to that found in the macrophage environment, evoked a shift to a starvation mode, as determined at transcriptional and proteomic levels. The metabolism alteration included gluconeogenesis and ethanol increases, activation of fatty acids and amino acid degradation, as strategies used by the pathogen to persist under this stress (Lima *et al.,* 2014).

As described above, studies have begun to elucidate the complex transcriptional and translational programs that *P. brasiliensis* uses to survive when exposed to host-like conditions (de Arruda Grossklaus *et al.* 2013; Lima *et al.* 2014). Tavares and co-workers (2007), showed that upon phagocytosis, *Paracoccidioides* regulates the expression of 152 genes, primarily associated with glucose and amino acid limitation, cell wall construction and oxidative stress (Tavares *et al.,* 2007). In this study, we assess the response of *Paracoccidioides* to macrophage phagocytosis, by employing high throughput proteomic analysis. The contribution of the intracellular macrophage activities towards the changes in protein profiles, as well as the importance of regulated proteins for survival of *Paracoccidioides* within macrophages had been inferred. Particularly, the study demonstrated that cytochrome c peroxidase knockdown resulted in decreased survival of *P. brasiliensis* inside macrophages.

MATERIAL AND METHODS

Fungal strains and growing conditions

The *P. brasiliensis* isolates *Pb*18 (Matute *et al.* 2006) and *Pb*339 (Matute *et al.* 2006) were used in this work in the yeast form. The yeast cells were inoculated in BHI medium containing glucose 4% (w/v) during 48 h at 36 ºC, under agitation.

Phagocytosis assays

J774.A1 macrophage (Rio de Janeiro Cell Bank –BCRJ/ UFRJ, accession number: 0121),cellline, derived from a reticulum cell sarcoma (Ralph *et al.,* 1975) were used for phagocytosis assays. The J774.A1cells were cultured in RPMI medium containing bovine fetal serum 10% (v/v) (Vitrocell Embriolife,) and MEM non-essential amino acid solution (Sigma Aldrich, Missouri, USA) at 36ºC and 5% CO_2 until complete confluence. The phagocytosis assay was performed in 12 wells polypropylene plate (Greinner Bio-One, USA). A total of 10^6J774 macrophages were plated per well and grown in RPMI medium during 24 hours at 36ºC and 5% CO_2 for adherence. After, a total of $5x10^6$ *Pb*18yeast cells per well were added to the macrophages, reaching a yeast:macrophages cell ratio 5:1, and incubated during 24 hours at 36 ºC and 5% CO_2in RPMI medium containing IFN- γ (50U per mL) (Sigma Aldrich, Missouri, USA). The control condition was obtained by incubating $5x10^6$ yeast cells per well in RPMI medium during 24 h at 36ºC and 5% CO_2.

Phagocytosis index and evaluation of phagolysosomes maturation

The phagocytosis index was evaluated by using a coverslip in a 6-wells polypropylene plate for macrophage adherence. After phagocytosis, the coverslip was fixed in 4% paraformaldehyde, stained with May Grunwald-Giemsa and analyzed by conventional microscopy. A total of 500 macrophages were quantified to determine the average number of adhered/internalized fungal cells (Weber *et al.,* 2012). Experiments were performed in biological triplicates.

The maturation of phagolysosomes was checked by using the Lysotracker probe red DND99 (Life Technologies Carlsbad, USA), according to the manufacturer instructions. Briefly, after phagocytosis the cells was washed 3 times with sterile water and recovered from 6-wells polypropylene plate with a scraper and marked with 75mM of the lysotracker probe. The assay was evaluated in a fluorescence microscope Axioscope A1 (Carl Zeiss) at 579/599 nm.

Obtainment of protein extracts

After the phagocytosis assay, the *P. brasiliensis* cells were washed 3 times with phosphate saline buffer (PBS) and the macrophages were lysed by adding sterile water. The supernatant from lyses was obtained and centrifuged at 8,000g. The obtained pellet was washed with water. The pellet was ressuspended in a solution containing 20 mM Tris-HCl, pH 8.8, 2 mM$CaCl_2$(da Fonseca *et al.*, 2001) and protein extraction was performed in Bead Beater equipment (BioSpec, Bartlesville, USA) in tubes containing 200-500 µm of acid washed glass beads (Sigma Aldrich, Missouri, USA).

Digestion of protein extracts and nano-ESI-UPLC-MS^E analyses

The obtained protein extracts were quantified by using the Bradford reagent (Sigma Aldrich, Missouri, USA) and bovine serum albumin (BSA) (Sigma Aldrich, Missouri, USA) as standard. A total of 100µg of each protein extract was used for trypsin digestion, as previously described (Murad & Rech 2012; Murad *et al.*, 2011). After, 10µL of 50mM ammonium bicarbonate pH 8.5 buffer was added to the samples. The samples were treated with0.2% RapiGEST SF Surfactant (v/v) (Waters, Milford, USA), and incubated in a dry bath at 80ºC during 15 min. The samples weretreated with 100mM DTT (GE Healthcare, Piscataway, USA) at 60ºC during 30 min and 300mMiodacetamide (GE Healthcare, Piscataway, USA) at room temperature during 30 min. After, 20 µL of trypsin(50 ng/mL) (Promega, Madison, USA) was added to digest the samples at 37ºC in a dry bath during 16 h.To cleave and precipitate the RapiGEST reagent, 20 µL of 5% of trifluoroacetic acid solution (TFA) (v/v) was added to the samples that were incubated during 90 min at 37ºC. The supernatants were dried in a speed vacuum (Eppendorf, Hamburg, Germany) during 5 h. All obtained peptides were suspended in 100µL of solution containing 20mM of ammonium formiate and 200fmol of PHB (Rabbit PhosphorylaseB) (Waters) (MassPREP^{TM} protein).

Nanoscale LC separation of tryptic peptides was performed by using a nanoACQUITY^{TM} system (WatersCorporation, Manchester, UK) equipped with a Symmetry C18 5µm, 180µm X 20mm precolumn and a XBrigde C18 5µm, 200µm X 50mm column and a 1,7µm, 100µm X 100mm analytical reversed-phase column (Waters). Samples were analyzed in three replicates.

Data processing and protein identification

The MS data obtained via UPLC-MS were processed and examined using the ProteinLynx Global Server (PLGS) version 2.4 (Waters Corporation, Manchester, UK), setting the minimum repeat rate for each protein in all replicates to 2. Proteins were identified using the *Paracoccidioides* database (http://www.broadinstitute.org/annotation/genome/ paracoccidioides_brasiliensis/MultiHome.html). In addition, a constitutive protein detected in all replicates and with a low variance coefficient was used to normalize the expression protein level in the samples. Protein and peptides tables generated by PLGS were merged and the dynamic range of the experiments, peptides detection type, and mass accuracy were calculated using the softwares MassPivot and SpotFire, for each condition as described in (Murad and Rech, 2012). Proteins that presented 20% of difference in the expression value when compared to control condition were considered regulated by the phagocytosis treatment.

Obtainment of cytochrome C peroxidase (CCP) knockdown mutant

The antisense-RNA (aRNA) strategy was used, as described (Menino *et al.,* 2012). Briefly, DNA from wild-type *Pb*339 exponentially growing yeast cells was obtained after cell rupture as described (Sambrook & Russel 2001). Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA) and the oligonucleotides: as*ccp*-sense 5´CCGCTCGAGCGGGATAAGGAAACTGGAACTGGAG 3´; as*ccp*-antisense, 5´GGCGCGCCCCTGAGAGTGACCACGCTG 3´ were synthesized to amplify aRNA from *Pb*WT cytochrome c peroxidase (*ccp*) DNA. Plasmid construction for aRNA gene repression and *A. tumefaciens*-mediated transformation (ATMT) of *Pb*339 wild type yeast cells (WT) were performed as previously described (Almeida *et al.*, 2009). Briefly, the amplified *Pbccp*-aRNA fragments were cloned flanked by the calcium-binding protein promoter region (P-*cbp-1*) of*Histoplasma capsulatum* and by the cat-B termination region (T-*cat-B*) of *Aspergillus fumigatus*. The pUR5750 plasmid containing the hygromycin (Hyg)-resistance gene (*hph)* from *Escherichia coli* flanked by the glyceraldehyde-3-phosphate dehydrogenase promoter region (P-*gapdh*) and the trpC termitation region (T-*trpC*) from *Aspergillus nidulans* was used as a parental binary vector to harbor the aRNA cassette within the transfer DNA (T-DNA). The constructed binary vector was introduced into *A. tumefaciens* LBA1100 ultracompetent cells by electroporation and used to perform the ATMT of *P. brasiliensis* yeast cells. The selection of *P. brasiliensis* transformants (*Pbccp*-aRNA) was performed in BHI solid media containing Hyg B (75 μg/ml) during 15 days of incubation at 36 ^{o}C. Randomly selected Hyg resistant transformants were tested for mitotic stability by subculturing the fungus three times in Hyg 75 μg/ml and three more times in Hyg 100 μg/ml. *P. brasiliensis* yeast cells were also transformed with the empty parental vector pUR5750 (EV) as a control during assays carried out in this study. Investigation of *Pbccp* gene expression was performed after consecutive subculturing by quantitative real time PCR.

Quantitative real-time PCR (qRT-PCR)

The evaluation of CCP silencing was performed by qRT-PCR. The total RNA from *Pb*339WT, *Pb*EV and *Pb*ccp-aRNA yeast cells were extracted by using Trizol (TRI Reagent, Sigma-Aldrich, St. Louis, MO) and mechanical cell rupture using Mini-Beadbeater (Biospec Products Inc., Bartlesville). After, *in vitro* reverse transcription (SuperScript^{TM} III First-Strand Synthesis SuperMix; Invitrogen^{TM}, Life Technologies) was performed and the cDNAs were submitted to qRT-PCR reaction by using SYBR green PCR master mix (Life Techonologies, Foster City, CA) in the StepOnePlus^{TM} real-time PCR system (Life Techonologies, Foster City, CA). The expression values were calculated by using the transcript encoding to *alpha tubulin* (XM_002796593) as the endogenous control. The oligonucleotides used in the qRT-PCR reaction were: tubulin sense: 5'-ATGAAACGGCAAATCCCACCA-3'; tubulin antisense 5'- ACAGTGCTTGGGAACTATACC -3'; ccp-sense: 5' CTTTGACGACCGCGAGATTG 3' and ccp-antisense 5' GACCGTTCCACTTTCTCCAG. The qRT-PCR reaction was performed in triplicate for each cDNA sample and a melting curve analysis was performed to confirm single PCR product. The relative standard curve was generated using a pool of cDNAs of all the conditions used serially diluted 1:5 until 1:125. Relative expression levels of transcripts of interest were calculated using the standard curve method for relative quantification (Bookout *et al.*, 2006).

Evaluation of *Pbccp*-aRNA sensitivity to oxidative stress

The *P. brasiliensis*cells strain *Pb*339 silenced with the antisense technology for cytochrome C peroxidase (*Pbccp*-aRNA) were tested for sensitivity to oxidative stress.Cells were cultured as described above. A total of 10^5 and 10^6 cels/mL were platted on Fava Netto's semisolid medium containing menadione 80 and 110 mM. *P. brasiliensis* wild-type cells strain *Pb*339 (WT) and *Paracoccidioides* transformed with the binary empty vector (EV) were used as control.The plates were incubated at 36 ºC during 7 days and colonies images were captured.

RESULTS

Yeast cells and macrophage co-culture

In order to determine the conditions to study the proteins of yeast cells differentially expressed upon macrophage interaction, we performed co-culture assays (see MM) using a macrophage-yeast cells ratio of 1:5. In order to evaluate the efficiency of the phagocytosis experiment, we measured the phagocytosis index by counting the total of adhered/phagocytosed yeast cells in 500 macrophages. The phagocytosis index obtained for the *Paracoccidioides* isolate *Pb*18 was 21.2% with a standard deviation of 0.53(data not shown).

Evaluation of phagolysosomes maturation

The phagocytosis was also evaluated by monitoring the phagolysosome maturation by using a lysosome marker, as depicted in Figure 1, Panels B and C. The phagolysosome maturation was observed by using the Lysotracker probe in the phagocytosis experiments by using *P. brasiliensis*isolate *Pb*18, demonstrating that the phagocytosis of *Paracoccidioides* yeast cells by the J774 macrophages induces phagolysosome maturation.

Proteomic analysis

A total of 426 differentially expressed proteins were identified in *P. brasiliensis* yeast cells derived from infected macrophages and are depicted in the Supplementary Tables 1 and 2. The resulting NanoUPLC-MSE protein and peptide data generated by the PLGS process are shown in Figures Supplementary 1, Supplementary 2 and Supplementary 3. The false positive rates of proteins from control and yeast cells derived from infected macrophages were 1.73% and 10.41%, respectively. The experiments resulted in 4,461 and 3,384 identified peptides, where 59% and 56% of these were obtained from peptide match type data in the first pass, and 7% and 6% from the second pass, to control and yeast cells derived from infected macrophages, respectively. A total of 17% and 18% of total peptides were identified by a missed trypsin cleavage, and in-source fragmentation rate of 8% and 10% to control and yeast cells derived from infected macrophages, respectively (Supplementary Figure 1).

Supplementary Figure 2 depicts the peptide parts per million error (ppm). A total of 95.67% and 95.77% of the peptides were detected with an error of less than 15 ppm of the total peptides identified, for control and yeast cells derived from infected macrophages, respectively. Supplementary Figure 3 shows the results obtained from dynamic range detection, indicating that a 3-log range and a good detection distribution of high and low molecular weights were obtained for both conditions. A fold change difference of 20% in the protein expression level were used to obtain the proteins up- or down-regulated, respectively, in comparison to control yeast cells. From the total, 181 proteins were positively regulated during the macrophage infection (Supplementary Table 1), while 245 proteins were down-regulated (Supplementary Table 2).

Concerning to up regulation (Supplementary Table 1) most of the proteins were related to aminoacid metabolism (15.5% of the total), energy (17.9% of the total), lipid metabolism (5% of the total) protein folding and degradation (10.5% of the total) and cell rescue and virulence (10.5% of the total). Regarding to the down regulated proteins, the most upregulated functional class was that of the protein synthesis (26.5%of the total), followed by aminoacid metabolism (9.8% of the total) and energy (9.3%). Supplementary Figure 3 depicts the percentage of functional classes in the upregulated proteins (Supplementary Figure 3A) and down regulated (Supplementary Figure 3B).

***P. brasiliensis* adapts to the macrophage milieu by a reprogramming of the metabolism to produce glucose and by inhibition of protein synthesis**

Proteomic data allow the suggestion of the molecular mechanisms used by *P. brasiliensis* to adapt to the macrophage environment. As depicted in Table 1, the fructose1,6 biphosphatase is induced, suggesting increase in gluconeogenesis, in order to increase glucose pool inside the cells. The precursors for glucose probably are provided by an increase in amino acid degradation (Table 1). Enzymes related to glutamate (glutamate dehydrogenase), alanine (alanine glyoxylate aminotransferase) and aspartate (aspartate aminotransferase) are induced resulting in an enrichment of the glucose precursors such as, pyruvate, fumarate and oxaloacetate. Additionally, the catabolism of glycogen seems to be induced by the increased expression of glycogen posphorylase and phosphoglucomutase. In addition, fatty acids are used as fuel for the fungal survival inside the phagocytes, as suggested by the induction of the enzymes enoyl CoA hydratase and 3-ketoacyl-CoA thiolase. Ethanol production is also increased, as suggested by the induction of pyruvate decarboxylase and alcohol dehydrogenase. The pyruvate for that probably is coming from amino acid degradation. This fact could lead to fungal survival inside the macrophage once ethanol production contributes to pathogenesis (Grahl *et al.,* 2011).The up regulated processes induced in *P. brasiliensis* upon internalization by macrophages are depicted in Figure 2.

As depicted in Table 2, the glycolytic specific enzymes phosphofructokinase 1, hexokinase and pyruvate kinase were all repressed, indicating that glucose is not used as an energy source, reinforcing that *P. brasiliensis* is facing a glucose poor environment inside macrophages. The inhibition on protein synthesis in the poor environment of macrophages is strongly suggested by the inhibition of a huge number of proteins related to the process (Supplementary Table 2 and Table 2).

It is important to highlight the overexpression of proteins related to autophagic protein degradation process, such as vacuolar aminopeptidase, carboxypeptidase Y and aspartyl protease (Table 3). The induction of the autophagic protein degradation is importante since provides a non-selective pathway for bulk turnover of the cytoplasmic components generating aminoacids during nutrient starvation (Takeshige *et al.,* 1992).

Up regulated proteins devoted to cell rescue and defense

The proteins related to cell defense, in response of *P. brasiliensis* to the macrophage milieu are depicted in Table 3, and Figure 3. Several effector proteins and enzymes are involved in the detoxification of the oxidative stress produced by the phagocytes. Superoxide is produced and intracellular superoxide dismutases are required for the breakdown of this radical. Superoxide detoxification generates H_2O_2. Among the enzymes capable of metabolizing hydrogen peroxide, *P. brasiliensis* induces cytochrome c peroxidase, as evidenced in Table 3 and Figure 3. The damage caused by ROS in enzymes localized in the cytoplasm and mitochondria can be reverted by thioredoxins.The enzyme gamma glutamyl transpeptidase catalyzes the transfer of the gamma-glutamyl moiety of glutathione to an acceptor that may be an amino acid, a peptide or water, a process leading to the depletion of glutamine and glutathione, resulting in increasing ROS production (Javed *et al.,* 2013). In addition to the detoxification mechanisms, as cited above, *P. brasiliensis* presents proteins putatively involved in sensing the presence of oxidants. The enhanced ROS production induced by gamma-glutamyltranspeptidase hasbeen described as a central factor in the activation of the oxidative stress cascades (Javed *et al.* 2013).

Evaluation of CCP silencing and knockdown mutant sensitivity to oxidative stress

Since the cytochrome C peroxidase (CCP) was up-regulated in *P. brasiliensis* during phagocytosis in macrophage cells, as evidenced in Table 3 and Figure 3, and due to the fact that the enzyme contributes do the antioxidant defense in fungi (Giles *et al.,* 2005) we decided to perform the generation of a mutant silenced strain to this gene, by using the antisense technology, as described (Menino *et al.* 2012; Bailão *et al.*, 2014).The cassette construction to obtain the CCP knockdown mutant is shown in the Figure 4, Panel A. The knockdown mutant was obtained in the *Pb*339 strain, which was demonstrated to be the most feasible for genetic transformation in our laboratory (Bailão *et al.* 2014). One construction was used to obtain the silenced mutants, as described above.The efficiency of the gene silencing from two *Paracoccidioides* transformants was evaluated by qRT-PCR, as depicted in Figure 4, Panel B. The silencing efficiency obtained for CCP knockdown mutants was around 50% (Figure 4, Panel B). The mutant strain obtained with the empty binary vector (EV) shows no significant difference in the *ccp* expression level when compared with the wild type strain (WT) (Figure 4, Panel B). In order to evaluate the sensibility of *ccp* silenced strains to oxidative stress, it was analyzed the growth of WT, EV and *Pbccp*-aRNA strains by using solid medium supplemented with menadione a free radical generator causing oxidative stress. It was observed that *ccp*-aRNA strain is more sensitive to menadione than WT and EV strains (Figure 5, Panel A) at the menadione 80 µM and 110 µM. The results strongly suggest CCP plays a role avoiding cell damage caused by oxidative stress.

The CCP silencing reduces the *P. brasiliensis* survival upon macrophage internalization

The *Pbccp*-aRNA was used to perform a phagocytosis assay in J774 macrophage cells. Control conditions were *Pb*339 wild type strain (WT) and *Pb*339 transformed with the empty binary vector (EV). The recovery of colony-forming units (CFU) after 24 hours of macrophage infection is depicted in the Figure 5, Panel B. The control strains WT and EV show no significant difference in the number of recovered CFU after the phagocytosis assay. Tthe number of CFU recovered from the CCP knockdown strain (*Pbccp*-aRNA) was severally reduced, suggesting the importance of the CCP protein during the *P. brasiliensis* phagocytosis in macrophage cells.

DISCUSSION

Macrophages are one of the most important types of phagocytes during *P. brasiliensis* infections, acting as a first line of defense. Although phagocytosis occurs by lung resident macrophages, *P. brasiliensis* is able to survive and multiply inside non activated macrophages; in contrast, lymphokine-activated macrophageswere fungicidal for the ingested fungus (Brummer *et al.*, 1989).

Previous work described the macrophage response to *P. brasiliensis* internalization by using microarray analysis. After 24 hours of phagocytosis the macrophages modulate positively the transcripts encoding chemokines and proteins for activation of T cells. Also, the macrophages up-regulate transcripts encoding for membrane proteins, such as lectins type receptors and selectins and transcripts encoding apoptose including caspases (Silva *et al*., 2008). In the other hand, the *P. brasiliensis* adaptation to macrophage internalization was evaluated by semi-quantitative RT-PCR and genes related to glyoxalate cycle and gluconeogenesis were up-regulated during the fungal internalization in murine macrophage (Derengowski *et al*., 2008).

A proteomic analysis of the response of *P. brasiliensis* upon interaction with macrophages has been carried out for the first time in this work. In the present work a total of 426 proteins were identified, as up or down-regulated in *P.brasiliensis* upon macrophage interaction. This number corresponds to proteins that had been detectedat least in two of three replicates. The metabolic changes detected in *P. brasiliensis* protein expression reflect how the yeast cells sense the macrophage hostile environment. Upon phagocytosis by macrophages *P. brasiliensis* activates responses related to the assimilation of alternative carbon sources than glucose, as well as the induction of proteins and enzymes related to the ROS detoxification. Proteins related to the Beta-oxidation of fatty acids, TCA cycle and most of the electron transport were induced, suggesting preferential use of fatty acids as a carbon fuel. Also, the key enzyme of gluconeogenesis was induced in addition to several enzymes of the amino acids catabolism, strongly suggesting the interconversion of them to pyruvate rendering glucose. In this way, *P. brasiliensis* might remodel its metabolism in order to recycle its own carbon-containing molecules. Under the same conditions we would expect the up-regulation of proteins of the glyoxylate cycle which enables microorganisms to grow on acetate or fatty acids as the sole carbon sources, a process that is an relevant adaptation of pathogenic microorganisms (Fernandez-Arenas *et al.,* 2007; Miramon *et al.,* 2012). The only induced enzyme belonging to the glyoxylate cycle was isocytrate lyase, while malate synthase is repressed in the same condition. The data suggest that gluconeogenesis and not the glyoxylate cycle perform an important role in the adaptive responses to phagocytosis.These data corroborating with findings to other fungi. *C. albicans* once phagocytized by macrophages or neutrophils decreases glycolytic enzymes and up regulates fatty-acid oxidation and glyoxylate cycle indicating that yeast is shifting to a starvation mode using alternative carbon sources (Fernandez-Arena *et al.*, 2007; Fradin *et al* 2005; Lorenz *et al.*, 2004). The induction of gluconeogenesis and amino acid degradation enzymes suggest that *P. brasiliensis* is using carbon backbones from amino acids to synthesize glucose and fatty acids as energy sources.

Phagocytic cells generate ROS (O^-_2, H_2O_2, OH) to eliminate fungal infections, and fungi are very efficient pathogens to respond to oxidative stress (Mansour & Levitz 2002). Synthesis of enzymes and antioxidant molecules ensure elimination of ROS (Thorpe *et al.,* 2004). In *P. brasiliensis*, a number of proteins for antioxidant defense systems have been described (de Arruda Grossklaus *et al.* 2013). In response to H_2O_2 generated oxidative stress, the fungus induces the accumulation of superoxide dismutases, cytochrome C peroxidase, thioredoxin and peroxisomal catalase (de Arruda Grossklaus *et al.* 2013; Chagas *et al.*, 2010). A transfer of a single electron to O_2 forms superoxide anion, whereas the transfer of two electrons yields peroxide. Superoxide dismutase scavenges superoxide radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen. Aldehyde dehydrogenases are critical in the detoxification of aldehydes. Oxidative stress and the consequent lipid peroxidation can lead to the generation of reactive aldehydes that can be oxidized to carboxylic acids by the aldehyde dehydrogenase superfamily (Esterbauer *et al.,* 1991). Thioredoxins are a family of small proteins that catalyze thiol-disulfide oxidoreductions by using redox-active cysteine residues (Holmgren 1985). More and more evidences confirms that H_2O_2 is primarily a signaling messenger (Rhee 2006). Thioredoxin has been shown to be involved in sensing of oxidative stress in *C. albicans* (da Silva Dantas *et al.,* 2010). The gene encoding the thioredoxin (TRX1) in this fungus, is up regulated during co-cultivation with neutrophils (Miramon *et al.* 2012). It has been proposed that non-active thiols of TRX1 play important roles in redox regulation of cell signaling via reversible oxidation in mammalian cells (Du *et al.*, 2013).

In addition to enzymes related to the fungus detoxification, *P. brasiliensis* seems to produce molecules that can be directed to the host damage. Gamma glutamyl transpeptidase plays a key role in synthesis and degradation of glutathione. The enzyme is a threonine N-terminal nucleophile hydrolase which catalyzes the transpeptidation and hydrolysis of the δ-glutamyl moiety of glutathione and related compounds. The reaction is accompanied by generation of ROS, as well as ammonia (Suzuki *et al.*, 1986). In the bacterium *Helicobacter bilis*, the enzyme induces enhanced ROS production as a central factor involved in the activation of the oxidative stress response cascades in host-infected cells (Javed *et al.* 2013). Arginase inhibits nitric oxide production by eukaryotic cells, as a strategy for *Helicobacter pylori* survival (Gobert *et al.*, 2001).

Additionally, upon macrophage infection, autophagy is presumably induced in *P. brasiliensis.* Carboxypeptidase Y, vacuolar aminopeptidases and aspartyl protease are involved in autophagy, a vacuolar trafficking pathway that targets subcellular constituents to the vacuole for degradation and recycling (Khalfan & Klionsky 2002). In *C. albicans*, aspartyl proteases are secreted proteins. The internalization of aspartyl proteases results in a partial permeabilization of lysosomal membranes activating the autophagy process (Wu *et al.*, 2013). Interestingly, the *P. brasiliensis*aspartyl protease (*Pb*SAP) is a secreted protease (Tacco *et al.,* 2009) and during macrophage infection the *Pb*SAP was detected in the fungal cell only in phagocyted yeast cells.

Taken together our results show that *P. brasiliensis* responds to several stress conditions when inside macrophages. Those insults include glucose deprivation and oxidative stress. The fungal response to glucose deprivation includes a metabolism shift from glucolysis to gluconeogenesis in which glucose precursors are provided by the catabolism of amino acids, as provided by proteomic analysis. We have also assessed the contribution of the fungal oxidative stress response mediated by cytochrome c peroxidase for the survival of *P. brasiliensis* by using knockdown strains. According to our data the enzymes depicts a relevant role for the fungus survival inside macrophages.

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Legends:

Figure 1: *In vitro* interaction of *P. brasiliensis* with macrophages. (A) Representative photo of macrophage cells infected with *Pb*18 after 24 hours. The cells are Giemsa stained and magnified 1000X. Black arrows evidence the yeast cells inside the macrophages **(B)** Bright field of non-stained macrophage cells after phagocytosis of *Pb*18 yeast cells magnified 400X. White arrows point vacuoles containing yeast cells. **(C)** The same field shown in panel B stained with Lysotracker probe to evidence the acidification of vacuoles containing *P. brasiliensis* yeast cells.

Figure 2: Molecular mechanism used by *P. brasiliensis* to survive inside macrophages. PYLG: glycogen phosphorylase; PGM: phosphoglucomutase; PGI: phosphoglucose isomerase; FPBase: fructose 1,6-biphosphatase; GAPDH glyceraldehyde 3-phosphate dehydrogenase; PGAM: phosphoglycerate mutase; ENO: enolase; PDC: pyruvate decarboxylase; ADH: alcohol dehydrogenase; PDH: pyruvate dehydrogenase; ICD: isocitrate dehydrogenase, SDH: succinate dehydrogenase, ECH: enoyl-CoA hydratase; KAT: 3-ketoacyl-CoA thiolase; AGXT: alanine glyoxylate aminotransferase; GOT: aspartate aminotransferase; HGD: homogentisate 1,2 dioxygenase; HDP: 4-hydroxyphenylpyruvate dioxygenase; ADSS: adenylosuccinate synthetase; ABAT: 4-aminobutyrate aminotransferase; GLUD: glutamate dehydrogenase; ALDH: aldehyde dehydrogenase; ASP: aspartyl protease; CXP: carboxypepetidase Y; APE: vacuolar aminopeptidase; ND: NADHubiquinone oxidoreductase; COX: cytochrome C oxidase; ATPase: ATP synthase. Up-regulated enzymes are shown in red.

Figure 3: *P. brasiliensis* response to oxidative stress upon macrophage internalization. (A) The *Paracoccidioides* protein response to ROS detoxification during macrophage infection includes the up-regulation of superoxide dismutases (SOD) and cytochrome c peroxidase (CCP) that detoxify ROS formed by macrophages. **(B)** Since the ROS formation can generate reactive aldehyde species (RAS) by lipid peroxidation, the up-regulation of aldehyde dehydrogenase (ALDH) can contribute to convert RAS into carboxylic acids. **(C)** The protein damage caused by ROS is repaired by thioredoxins (TRX) that are up-regulated during macrophage infection. **(D)** The up-regulation of gamma glutamyltranspeptidase (GGT) can also contribute to *P. brasiliensis* response to internalization since can increase the ROS production by the fungus to activate the oxidative stress response in the host.

Figure 4: Generation of cytochrome C peroxidase silenced mutant (*Pbccp*-aRNA) and evaluation of silencing efficiency (A) Schematic representation of the DNA cassete used to silence the cytochrome C peroxidase protein. The DNA cassete cloned in pUR5750 binary vector is composed by: (i) a silencing cassette containing a promoter region of the *Histoplasma capsulatum* CBP1 gene (CBP1) and termination region of the *Aspergillus fumigatus* cat-B gene (T-cat-B) flanking the CCP antisense fragment (CCP-AS) and (ii) a resistance cassette containing the glyceraldehyde-3-phosphate dehydrogenase promotor gene (GAPDH) and the trpC termination region (trpC) from *Aspergillus nidulans* flanking the hygromycin-resistance gene (HPH) from *Escherichia coli.* **(B)** Relative quantification performed by Real-Time quantitaive PCR to confirm the CCP silencing. WT: wild type yeast cells (*Pb*339 strain); EV: *Y*east cells (*Pb*339 strain) containing the empty vector with no CCP-AS; *Pbccp*-aRNA1 and *Pbccp*-aRNA2: Colonies from yeast cells (*Pb*339 strain) containing the cassette with the*ccp* antisense fragment.

Figure 5: Evaluation of the *Pbccp*-aRNA silenced mutant sensitivity to oxidative stress and survival into macrophages. (A) The *Pbccp*-aRNA sensitivity to oxidative stress was performed by using menadione 80 µM and 110 µM. *P. brasiliensis* WT and EV was used as control. **(B)** Phagocytosis assay. WT: wild type (*Pb*339 strain); EV: Yeast cells (*Pb*339 strain) containing the empty vector without CCP-AS; *Pbccp*-aRNA1: Colony 1 of yeast cells (*Pb*339 strain) containing the cassete whith CCP-AS fragment.

Supplementary Figure 1. Peptide detection type to control and *Paracoccidioides brasiliensis* derived from infected macrophage. PepFrag 1 and PepFrag 2 correspond to the peptide matches when compared to database by PLGS, Var Mod corresponds to variable modifications, In Source corresponds to fragmentation that occurred on ionization source, MissedCleavage indicates the missed cleavage performed by trypsin and Neutral loss H_2O and NH_3 correspond to water and ammonia precursor losses; Control **(A)** and **(B)** *Paracoccidioides brasiliensis* derived from infected macrophage **(B)**. The Pep Frag parameters should be predominant, in contrast to missed cleavage which should not reach 20%.

Supplementary Figure 2. Indicate the number of identified peptides in a 15 ppm error range. A total of 99.67% and 95.77% of identified peptides were detected in both samples. Control **(A)** and *Paracoccidioides brasiliensis* derived from infected macrophage **(B)**, respectively.

Supplementary Figure 3. Detection dynamic range. Graphs for control **(A)** and *P. brasiliensis* derived from infected macrophage **(B)** are shown. Regular, reverse, and standard proteins were indicated by red/ square, blue/ square and yellow/ square colors, respectively. The protein with thelower coefficient ofvariancebetween samples, arrows indicate (conserved hypothetical protein, accession PADG_01220, Fmol CV 0.08) was used to normalize the expression data and compare the control and *P. brasiliensis* derived from infected macrophage.

Table 1 – Selected up-regulated proteins in *P. brasiliensis* yeast cells during macrophage infection, related to alternative carbon metabolism

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Gluconeogenesis				
PADG_01706	fructose 1 6 bisphosphatase	5195,62	1,54	1
PADG_00451	glucose 6 phosphate isomerase	2086,32	1,26	1
PADG_04059	Enolase	60972,12	1,84	1
PADG_02411	glyceraldehyde 3 phosphate dehydrogenase	72801	1,97	1
PADG_06358	phosphoglycerate mutase family protein	569,7	*	*
Anaerobic metabolism				
PADG_04701	alcohol dehydrogenase	759,02	1,34	1
PADG_02271	alcohol dehydrogenase	874,63	2,39	1
PADG_00714	pyruvate decarboxylase	1628,1	1,70	1
Glycogen metabolism				
PADG_02145	glycogen phosphorylase	1335,73	1,30	1
PADG_00681	Phosphoglucomutase	2716,46	2,46	1
Tricarboxilic acid cycle				
PADG_06494	dihydrolipoyl dehydrogenase	9770,13	2,46	1
PADG_00246	pyruvate dehydrogenase E1 component subunit beta	3817,45	1,28	1
PADG_07213	pyruvate dehydrogenase protein X component	2020,04	1,32	1
PADG_04249	isocitrate dehydrogenase	1057,26	1,21	1
PADG_00052	succinate dehydrogenase flavoprotein subunit	1120,04	1,60	1
PADG_07475	succinate dehydrogenase flavoprotein subunit	120,14	*	*
PADG_08013	succinate dehydrogenase iron sulfur subunit	665,89	*	*
Beta-oxidation of fatty acid				
PADG_03194	3 ketoacyl CoA thiolase B	1526,96	1,23	1
PADG_01209	enoyl CoA hydratase	11615,45	1,84	1
Amino acid degradation				
PADG_03020	alanine glyoxylate aminotransferase	1597,6	1,93	1
PADG_01621	aspartate aminotransferase	768,6	*	*
PADG_08466	homogentisate 1 2 dioxygenase	1626,28	1,25	1
PADG_08468	4 hydroxyphenylpyruvate dioxygenase	9595,33	2,10	1
PADG_00832	adenylosuccinate synthetase	1083,69	1,27	1
PADG_02214	4 aminobutyrate aminotransferase	1086,24	1,79	1
PADG_04516	NADP specific glutamate dehydrogenase	1485,79	5,00	1
PADG_05081	aldehyde dehydrogenase	5771,94	1,22	1

[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] Fold change of the protein expression ratios obtained from *P. brasiliensis* yeast cells during macrophage infection : *P. brasiliensis* control condition**.** Proteins with fold change of 20% were considered regulated during macrophage infection.

[3] *p*-value of the fold change. It was considered statistically significant the p-values less than 0,05 and higher than 0,95.

Table 2 – Selected down-regulated proteins in *P. brasiliensis Pb1*8 yeast cells during macrophage infection

Accession number^1	**Protein description**	**Score**	**Fold change^2**	**p value^3**
Glycolysis				
PADG_01896	PADG_01896	PADG_01896	PADG_01896	PADG_01896
PADG_03813	PADG_03813	PADG_03813	PADG_03813	PADG_03813
PADG_00192	PADG_00192	PADG_00192	PADG_00192	PADG_00192
PADG_01278	PADG_01278	PADG_01278	PADG_01278	PADG_01278
PADG_06906	PADG_06906	PADG_06906	PADG_06906	PADG_06906
PADG_00668	PADG_00668	PADG_00668	PADG_00668	PADG_00668
Protein synthesis				
PADG_07888	eukaryotic translation initiation factor 5A	4811,73	0,83	0,03
PADG_04057	translation initiation factor eIF3	4000,27	*	*
PADG_00932	translation initiation factor eIF3	478,38	*	*
PADG_01891	translation initiation factor RLI1	656,19	*	*
PADG_06110	translation factor SUI1	3845,67	*	*
PADG_00692	elongation factor 1 alpha	25477,84	0,23	0
PADG_02759	ribosome recycling factor domain-containing protein	1902,46	*	*
PADG_02752	116 kDa U5 small nuclear ribonucleoprotein component	110,37	0,53	0
PADG_04307	mRNA binding post transcriptional regulator Csx1	2870,54	0,71	0
PADG_04730	nascent polypeptide associated complex subunit alpha	1887,05	*	*
PADG_02896	elongation factor 1 beta	27699,38	0,36	0
PADG_06265	elongation factor 1 gamma 1	21636,54	0,41	0
PADG_08125	elongation factor 2	8384,21	0,45	0
PADG_03431	putative tRNA-binding protein	533,64	*	*
PADG_03440	prolyl tRNA synthetase	454,75	*	*
PADG_01558	histidyl tRNA synthetase	1005,73	*	*
PADG_02484	valyl tRNA synthetase	844,82	*	*
PADG_03689	tyrosyl tRNA synthetase	1451,04	*	*
PADG_05848	glycyl tRNA synthetase	600,14	*	*
PADG_05897	seryl tRNA synthetase	2707,68	*	*
PADG_08472	lysyl tRNA synthetase	857,74	*	*
PADG_04962	aspartyl tRNA synthetase	2972,06	0,54	0
PADG_00785	ribosomal protein S15	1042,95	*	*
PADG_07583	ribosomal protein S21E	11069,39	0,84	0
PADG_01654	ribosomal protein S6 PA	6916,45	0,74	0
PADG_02142	ribosomal protein L5	13042,87	0,75	0
PADG_04449	ribosomal protein L23e	2224,35	0,78	0
PADG_01267	30S ribosomal protein S17P	4145,94	0,72	0
PADG_01503	37S ribosomal protein Rsm24	553,96	*	*
PADG_03315	40S ribosomal protein S4	5778,31	0,72	0
PADG_06838	40S ribosomal protein S5	8692,07	0,84	0
PADG_07863	40S ribosomal protein S8	7473,32	0,77	0
PADG_03326	40S ribosomal protein S9	8370,36	0,71	0
PADG_04866	40S ribosomal protein S10 A	3053,9	*	*
PADG_01427	40S ribosomal protein S12	3182,33	0,69	0
PADG_07685	40S ribosomal protein S13 1	8064,19	0,78	0
PADG_02445	40S ribosomal protein S15	10623,49	0,39	0
PADG_00333	40S ribosomal protein S16	4491,59	0,76	0
PADG_00354	40S ribosomal protein S17	12836,03	0,79	0
PADG_06502	40S ribosomal protein S20	7199,8	0,48	0
PADG_06680	40S ribosomal protein S22	4014,56	0,83	0
PADG_06599	40S ribosomal protein S25	539,48	*	*
PADG_08605	40S ribosomal protein S28	4991,9	*	*
PADG_00784	40S ribosomal protein S0	8686,2	0,82	0

PADG_04030	60S acidic ribosomal protein P0	7617,59	0,68	0
PADG_02249	60S ribosomal protein L2	7260,73	0,70	0
PADG_07173	60S ribosomal protein L3	2294,24	0,77	0
PADG_05721	60S ribosomal protein L4 A	4738,24	0,70	0
PADG_01387	60S ribosomal protein L7	2449,69	0,68	0
PADG_04848	60S ribosomal protein L8 B	14038,4	0,63	0
PADG_02828	60S ribosomal protein L10a	1216,32	0,59	0
PADG_03778	60S ribosomal protein L10	3824,79	0,68	0
PADG_07803	60S ribosomal protein L12	9790,37	0,63	0
PADG_03856	60S ribosomal protein L15	8385,96	0,73	0
PADG_00514	60S ribosomal protein L16	3425,75	0,73	0
PADG_06726	60S ribosomal protein L17	2919,98	0,66	0
PADG_05264	60S ribosomal protein L19	4454,72	0,68	0
PADG_03873	60S ribosomal protein L20	4368,79	0,70	0
PADG_03325	60S ribosomal protein L21 A	9303,96	0,77	0
PADG_05883	60S ribosomal protein L23a	11162,32	0,82	0
PADG_00612	60S ribosomal protein L27 A	8874,16	0,69	0
PADG_05939	60S ribosomal protein L27a	489,74	0,78	0,02
PADG_03781	60S ribosomal protein L30	10975,43	0,83	0
PADG_01083	60S ribosomal protein L32	6304,8	0,78	0
PADG_01026	60S ribosomal protein L43	7369,92	0,66	0

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[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] Fold change of the protein expression ratios obtained from *Paracoccidioides* yeast cells during macrophage infection : *Paracoccidioides* control condition**.** Proteins with fold change of 20% were considered regulated in *Paracoccidioides* protein extract during macrophage infection.

[3] *p*-value of the fold change. It was considered statistically significant the p-values less than 0,05 and higher than 0,95.

* Proteins detected in *P. brasiliensisPb*18 only in the control condition.

Table 3 – Up regulated proteins related to cell rescue and defense in *Paracoccidioides Pb*18 yeast cells during macrophage infection

Accession number[1]	**Protein description**	**Score**	**Fold change**[2]	**p value**[3]
PADG_01479	Gamma glutamyltranspeptidase	577,9	1,95	1
PADG_07460	Vacuolar aminopeptidase	693,28	2,34	1
PADG_06314	Carboxypeptidase Y	504,42	3,19	1
PADG_00634	Aspartyl protease	452,93	*	*
PADG_07749	Protoplast secreted protein - Y20	38131,91	1,55	1
PADG_05183	Mitochondrial monothiol glutaredoxin 5	1304,22	J774	*
PADG_02764	Thioredoxin-like protein	2118,45	2,86	1
PADG_03161	Thioredoxin	647,52	*	*
PADG_05504	Mitochondrial thioredoxin	46080,04	1,22	1
PADG_03163	Mitochondrial cytochrome c peroxidase	6455,72	1,68	1
PADG_01954	Mitochondrial Fe/Mnsuperoxide dismutase	1207,88	1,42	1
PADG_07418	Cu/Zn superoxide dismutase	6827,38	1,77	1
PADG_05081	Aldehyde dehydrogenase	5771,94	1,22	1

[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] Fold change of the protein expression ratios obtained from *Paracoccidioides* yeast cells during macrophage infection : *Paracoccidioides* control condition**.** Proteins with fold change of 20% were considered regulated in *Paracoccidioides* protein extract during macrophage infection.

[3] *p*-value of the fold change. It was considered statistically significant the p-values less than 0,05 and higher than 0,95.

* Proteins detected in *P. brasiliensis Pb*18 only during macrophage infection.

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Figure 1

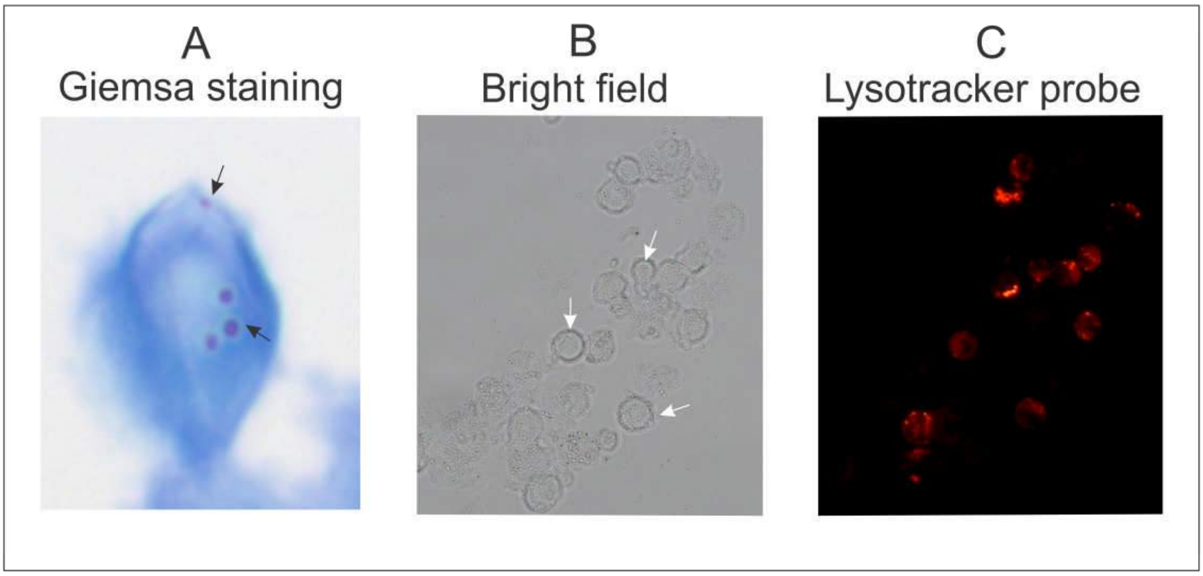
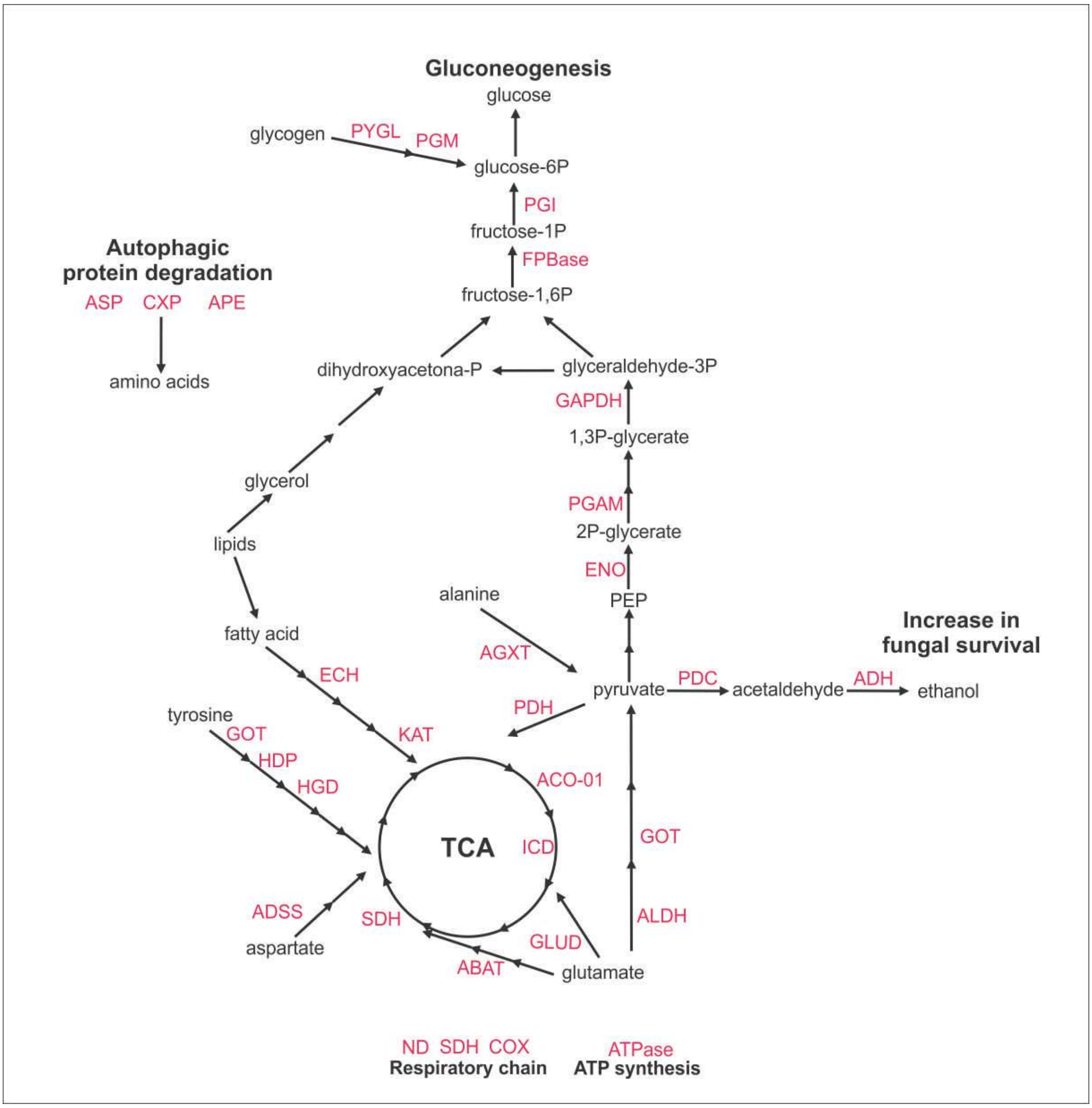


Figure 2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glycogen
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FPBase
fructose-1,6P
Autophagic protein degradation
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CXP
APE
amino acids
dihydroxyacetona-P
glyceraldehyde-3P
GAPDH
1,3P-glycerate
glycerol
PGAM
2P-glycerate
lipids
ENO
PEP
alanine
fatty acid
AGXT
Increase in fungal survival
ECH
PDC
ADH
pyruvate
acetaldehyde
ethanol
PDH
tyrosine
GOT
KAT
HDP
ACO-01
HGD
TCA
ICD
GOT
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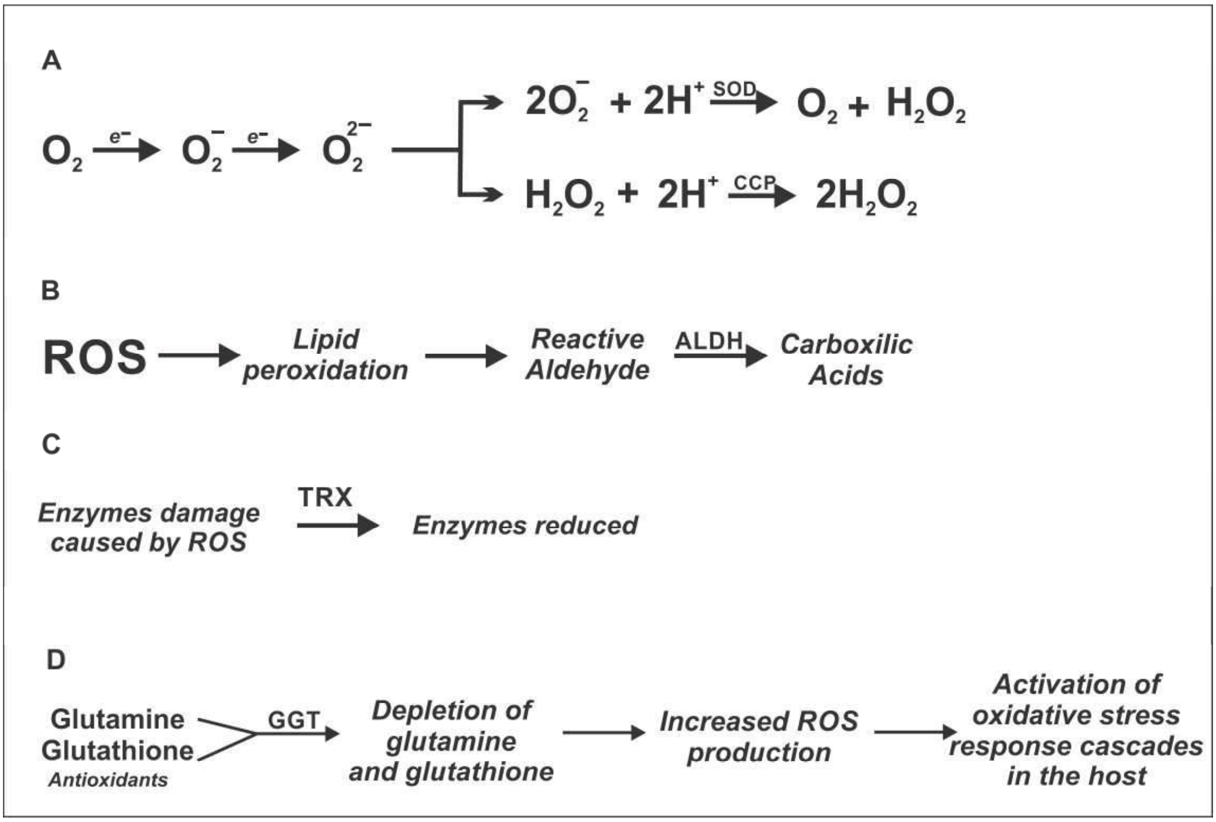
COX
Respiratory chain
ATPase
ATP synthesis

Figure 3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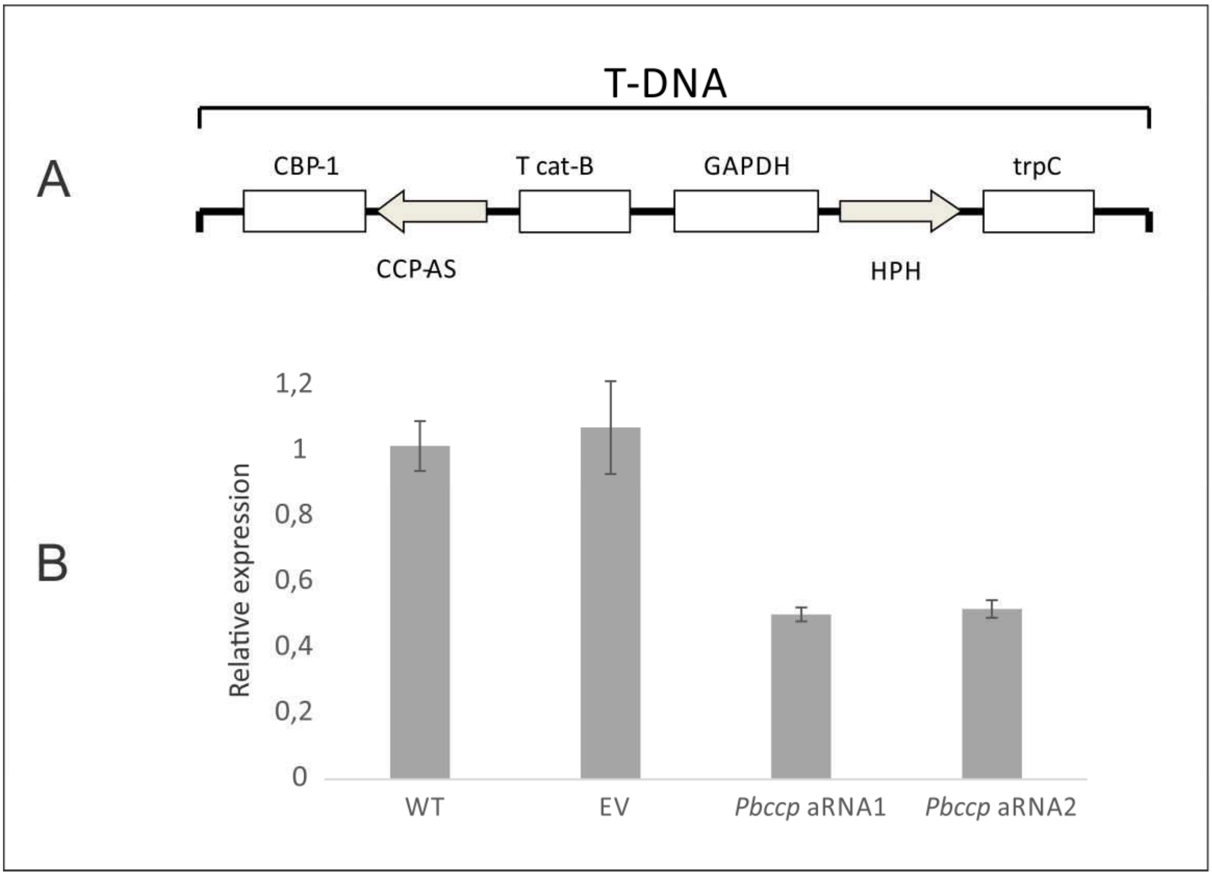
O2 e− O2− e− O2 2−
2O2− + 2H+ SOD O2 + H2O2
H2O2 + 2H+ CCP 2H2O2
B
ROS
Lipid peroxidation
Reactive Aldehyde
ALDH
Carboxilic Acids
C
Enzymes damage caused by ROS
TRX
Enzymes reduced
D
Glutamine
Glutathione
Antioxidants
GGT
Depletion of glutamine and glutathione
Increased ROS production
Activation of oxidative stress response cascades in the host

Figure 4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CBP-1
T cat-B
GAPDH
trpC
CCP-AS
HPH
B
Relative expression
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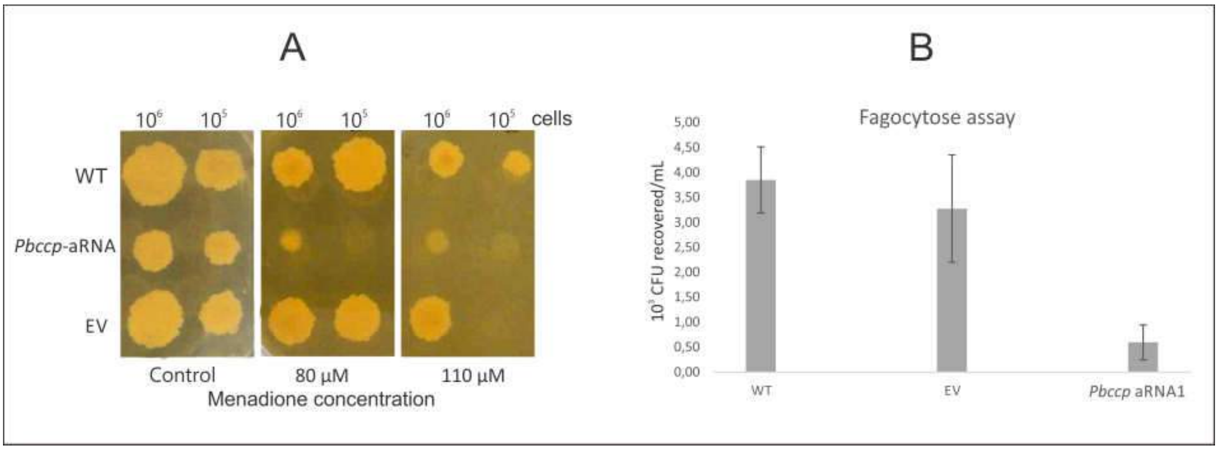
0,8
0,6
0,4
0,2
0
WT
EV
Pbccp aRNA1
Pbccp aRNA2

Figure 5


A
10^6
10^5
10^6
10^5
10^6
10^5
cells
WT
Pbccp-aRNA
EV
Control
80 µM
110 µM
Menadione concentration
B
Fagocytose assay
10^3 CFU recovered/mL
5,00
4,50
4,00
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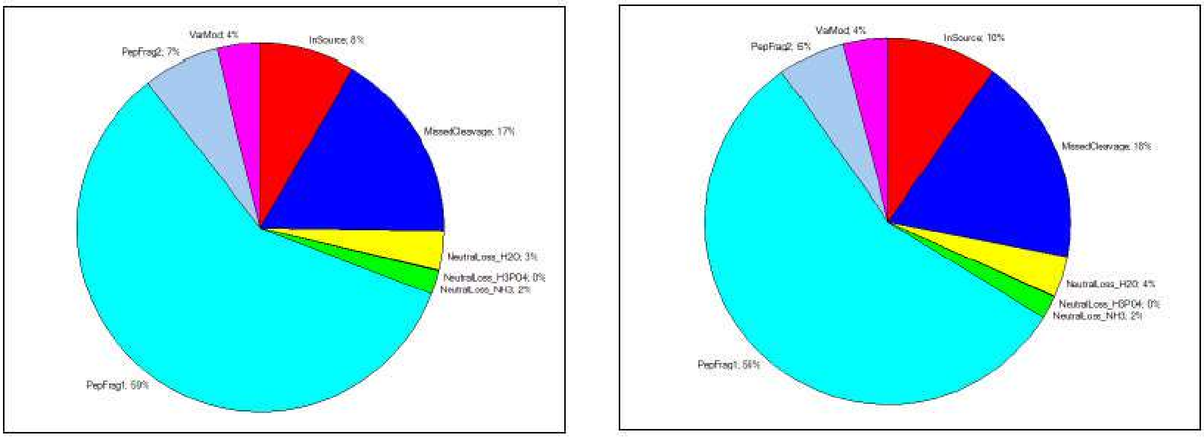
1,00
0,50
0,00
WT
EV
Pbccp aRNA1

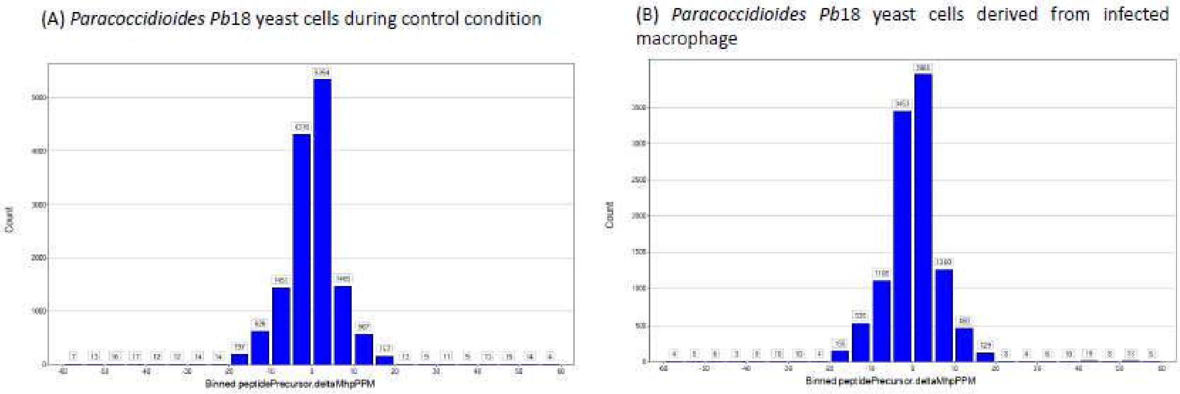
Supplementary Figure 1



(A) *Paracoccidioides Pb*18 yeast cells during control condition

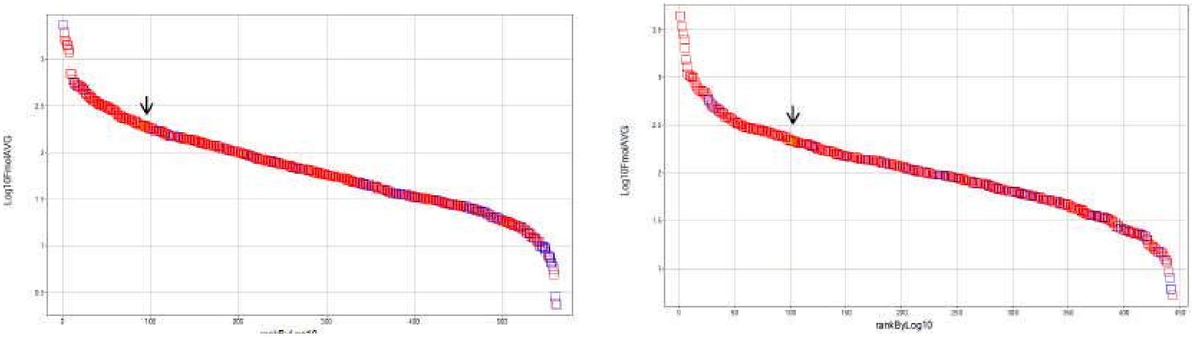
(B) *Paracoccidioides Pb*18 yeast cells derived from infected macrophage

Supplementary Figure 2


(A) *Paracoccidioides Pb*18 yeast cells during control condition

(B) *Paracoccidioides Pb*18 yeast cells derived from infected macrophage

Supplementary Figure 3



(A) *Paracoccidioides Pb*18 yeast cells during control condition

(B) *Paracoccidioides Pb*18 yeast cells derived from infected macrophage

Supplementary table 1 – Up-regulated proteins of *P. brasiliensis* during macrophage infection in J774 cells.

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Amino acid metabolism				
PADG_00366	NAD dependent epimerase dehydratase family protein	1010,24	1,22	1
PADG_01564	Methyl malonate semi aldehyde dehydrogenase	1698,87	1,35	1
PADG_02214	4 aminobutyrate aminotransferase	1086,24	1,79	1
PADG_04570	Branched chain amino acid aminotransferase	572,97	1,80	1
PADG_01621	Aspartate aminotransferase	768,6	*	*
PADG_04516	NADP specific glutamatedehydrogenase	1485,79	5,00	1
PADG_05085	Delta 1 pyrroline 5 carboxylate dehydrogenase	3968,2	1,57	1
PADG_00637	Arginase	458,37	4,31	1
PADG_03871	Urease	514,17	1,31	1
PADG_02777	Threonine synthase	595,36	*	*
PADG_06252	1 2 dihydroxy 3 keto 5 methyl thiopentene dioxygenase	754,99	*	*
PADG_08376	Aspartate semi aldehyde dehydrogenase	1013,55	1,43	1
PADG_00210	Glycine dehydrogenase	1743,71	1,25	1
PADG_00405	Choline dehydrogenase	544,21	1,38	1
PADG_03020	Alanine glyoxylate amino transferase	1597,6	1,93	1
PADG_08468	4 hydroxy phenyl pyruvate dioxygenase	9595,33	2,10	1
PADG_02914	Amino methyl transferase	3507,39	1,21	1
PADG_05492	NADP dependent L serine L allo threonine dehydrogenase ydfG	1951,52	1,21	1
PADG_02728	Sulfite oxidase	4920,6	1,23	1
PADG_07907	Acetolactate synthase	1269,3	2,14	1
PADG_08466	Homogentisate 1 2 dioxygenase	1626,28	1,25	1
PADG_04854	Anthranilate synthase component	349,36	1,58	1
PADG_06429	Ketol acid reducto isomerase	1929,1	1,34	1
PADG_03514	2 oxoisovalerate dehydrogenase subunit alpha	455,86	1,23	1
PADG_06671	3 isopropyl malatedehydrogenase A	525,06	3,53	1
PADG_07370	Methyl crotonoylCoA carboxylase beta chain	1362,12	1,22	1
PADG_03114	Phospho 2 dehydro 3 deoxyheptonate aldolase	862,15	*	*
PADG_00832	Adenylo succinate synthetase	1083,69	1,27	1
Nitrogen and sulfur metabolism				
PADG_04819	Nitrilase	4736,89	1,25	1
PADG_06490	Formamidase	4525,27	*	*
PADG_05837	Glycoprotein FP21	3841,88	1,35	1
Nucleotide and nucleoside metabolism				
PADG_02183	ADP ribose pyrophosphatase	2107,17	*	*
PADG_08066	Purine nucleoside phosphorylase	1018,83	*	*
PADG_05225	Orotidine 5 phosphate decarboxylase	1913,43	*	*
PADG_00331	Uricase	746,21	*	*
PADG_04828	Adenylo succinate lyase	2431,85	2,34	1
PADG_07524	Nucleoside diphosphate kinase	44024,86	1,39	1
PADG_02561	ATPase alpha subunit	13537,11	2,69	1
Signal transduction				
PADG_06273	Calcineurin subunit B	976,11	*	*
PADG_02017	Calmodulin	2829,23	4,10	1
PADG_00282	GTP bindingprotein SAS1	1656,09	*	*
PADG_03522	Phosphorylase family protein	2028,21	3,32	1
DNA repair				
PADG_02683	DNA repair protein RAD23	1882,35	1,86	1

Accession number[1]	**Protein description**	**Score**	**Fold change[2]**	**p value[3]**
C-compound and carbohydrate metabolism				
PADG_04939	SuccinylCoA 3 ketoacidc enzyme A transferase subunit B	1823,47	2,25	1
PADG_05855	Lactonohydrolase	684,03	2,39	1
PADG_00912	UDP galactopyranosemutase	3906,35	2,29	1
PADG_07615	Glucan 1 3 beta glucosidase	660,12	*	*
Pentose phosphate pathway				
PADG_07771	6 phosphoglucono lactonase	1595,8	1,84	1
Glycolysis				
PADG_00451	Glucose 6 phosphate isomerase	2086,32	1,26	1
PADG_04059	Enolase	60972,12	1,84	1
PADG_02411	Glyceraldehyde 3 phosphate dehydrogenase	72801	1,97	1
PADG_06358	Phospho glycerate mutase family protein	569,7	*	*
Gluconeogenesis				
PADG_01706	Fructose 1 6 bisphosphatase	5195,62	1,54	1
Anaerobic metabolism				
PADG_04701	Alcohol dehydrogenase	759,02	1,34	1
PADG_02271	Alcohol dehydrogenase	874,63	2,39	1
PADG_00714	Pyruvate decarboxylase	1628,1	1,70	1
Tricarboxilic acid cycle				
PADG_06494	Dihydro lipoyl dehydrogenase	9770,13	2,46	1
PADG_00246	Pyruvate dehydrogenase E1 component subunit beta	3817,45	1,28	1
PADG_07213	Pyruvate dehydrogenase protein X component	2020,04	1,32	1
PADG_04249	Isocitrate dehydrogenase	1057,26	1,21	1
PADG_00052	Succinate dehydrogenase flavoprotein subunit	1120,04	1,60	1
PADG_07475	Succinate dehydrogenase flavoprotein subunit	120,14	*	*
PADG_08013	Succinate dehydrogenase iron súlfur subunit	665,89	*	*
Glyoxylate cycle				
PADG_01483	Isocitrate lyase	2004,06	1,63	1
Methylcitrate cycle				
PADG_04709	Mitochondrial 2 methyl isocitrate lyase	645,58	1,30	1
PADG_04718	2 methylcitrate dehydratase	20965,98	1,34	1
Glycogen catabolism				
PADG_02145	Glycogen phosphorylase	1335,73	1,30	1
PADG_00681	Phospho glucomutase	2716,46	2,46	1
Electron transport and respiration				
PADG_03559	Cytochrome b5	2557,41	*	*
PADG_00171	Cytochrome b2	774,5	1,46	1
PADG_05750	Cytochrome c oxidase polypeptide VI	4509,55	1,35	1
PADG_01366	NADH-ubiquinone oxido reductase	3474,65	*	*
PADG_02745	NADH ubiquinone oxido reductase	1333,29	*	*
ATP synthesis				
PADG_00688	Mitochondrial F1F0 ATP synthase subunit Atp14	5922,63	*	*
PADG_04729	ATP synthase D chain mitochondrial	826,29	2,34	1
PADG_07813	ATP synthase gammachain	1496,76	2,01	1
PADG_08349	ATP synthase subunit beta	25936,79	2,66	1
PADG_07789	ATP synthase delta chain	1261,26	4,85	1
Beta-oxidation of fatty acid				
PADG_03194	3 ketoacyl CoA thiolase B	1526,96	1,23	1
PADG_01209	Enoyl CoA hydratase	11615,45	1,84	1

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Lipid metabolism				
PADG_02751	Acetyl CoA acetyltransferase	1733,16	1,21	1
PADG_07031	Hydroxy methyl glutarylCoA lyase	453,69	1,43	1
PADG_01363	AcylCoA binding protein	3006,95	1,95	1
PADG_06382	AcetylCoA acetyltransferase	4214,67	3,19	1
PADG_05310	Leukotriene A 4 hydrolase	692	J774	*
PADG_07699	S formyl glutathione hydrolase	2590,55	*	*
PADG_08018	Glycerol 3 phosphate dehydrogenase	594,46	*	*
PADG_05783	Farnesyl pyrophosphate synthetase	3647,17	1,30	1
PADG_04687	3 beta hydroxysteroid dehydrogenase	1928,12	1,16	0,99
Biosynthesis of vitamins and cofactors				
PADG_00443	Dihydropteroate synthase	1358,83	1,27	1
PADG_05822	Pyridoxine biosynthesis protein PDX1	8184,32	1,84	1
PADG_01886	Adenosyl homocysteinase	29827,71	2,39	1
PADG_05474	Thiamine pyrophosphokinase	477,33	3,25	1
PADG_00607	Riboflavin synthase alpha chain	1575,84	*	*
PADG_02981	ThiJPfpI familyprotein	11388,36	1,25	1
PADG_04603	Spermidine synthase	2288,22	1,77	1
PADG_08108	Coproporphyrinogen III oxidase	1670,3	*	*
PADG_08328	Cobalaminin dependent synthase	7405,12	2,20	1
Mitotic cell and cell cycle control				
PADG_03905	Proliferating cell nuclear antigen	7967,5	3,49	1
PADG_05615	Ran specific GTPase activating protein	13103,23	1,67	1
PADG_03073	Nuclear movement protein nudC	4613,94	1,84	1
Cell growth and morphogenesis				
PADG_08615	Tropomyosin	10023,92	3,32	1
PADG_00422	Actin cytoskeleton protein VIP1	2903,23	1,28	1
PADG_06691	Alpha 1,4 amylase	532,98	*	*
PADG_08350	1,4 alpha glucan branching enzyme	598,52	4,48	1
Transcriptional control				
PADG_00872	Histone H4	5533,47	*	*
PADG_00873	Histone H3	2942,48	*	*
Transcription				
PADG_05393	mRNA decapping hydrolase	676,79	2,89	1
PADG_02825	Ribonucleoprotein LSM domain	1707,02	*	*
Protein synthesis				
PADG_04735	Signal recognition particle protein	579,28	*	*
PADG_01079	Translation initiation factor 4B	450,49	*	*
PADG_02555	RNA binding domain containing protein	3684,84	1,22	1
PADG_04657	Nascent polypeptide associated complex subunit beta	954,52	*	*
PADG_01914	Ribosomal protein L35	2949,8	*	*
PADG_04315	40S ribosomal protein S24	3476,38	1,48	1
PADG_02056	50S ribosomal protein L12	4892,1	1,23	0,98
PADG_02888	60S ribosomal protein L6	4366,53	1,26	1
PADG_05338	60S ribosomal protein L18 B	9565,45	1,32	1
PADG_07924	60S ribosomal protein L24	2095,76	*	*
PADG_05025	60S ribosomal protein L126	1190,56	1,34	0,99
PADG_00046	60s ribosomal protein mitochondrial precursor	1153,31	*	*
PADG_08244	60S acidic ribosomal protein P1	28689,8	3,53	1
PADG_02446	60S acidic ribosomal protein P2	15620,39	3,97	1
PADG_01406	Phenyl alanyl tRNAsynthetase beta chain	717,35	1,22	0,99
PADG_06249	Glutaminyl tRNA synthetase	956,71	2,10	1
PADG_04288	L PSP endoribonuclease family protein Hmf1	37089,53	2,12	1

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Protein folding and stabilization				
PADG_07599	Peptidyl proly lisomerase B	1623,05	1,54	1
PADG_01852	Small glutamine rich tetratrico peptide repeat containing protein	2516,5	2,34	1
PADG_00759	Prefoldin subunit 4	2058,89	*	*
Protein degradation				
PADG_00615	Proteasome subunit alpha type 6	2488,98	*	*
PADG_00995	Ubiquitin	12786,32	1,25	1
PADG_07891	Ubiquitin	7292,33	*	*
PADG_06766	Mitochondrial processing peptidase subunit beta	686,78	1,54	1
PADG_03221	Saccharolysin	793,9	1,32	1
PADG_04076	Proteasome component C11	626,42	1,68	1
PADG_05922	Cytosolic non specificdipeptidase	4498,78	1,70	1
PADG_05820	Xaa Pro aminopeptidase	619,84	2,56	1
PADG_02421	Insulin degrading enzyme	366,54	*	*
PADG_05193	Xaa Pro aminopeptidase	1579,76	*	*
PADG_08442	Proteasome component Y13	574,28	1,58	1
PADG_07190	Proteasome component Y7	1806,88	1,51	1
PADG_03965	Proteasome component Pre4	682,25	1,55	1
PADG_03982	Proteasome component C1	1025,8	1,63	1
PADG_03727	Proteasome component PUP1	853,93	2,41	1
PADG_03680	Proteasome component PRE2	1212,62	*	*
Cell Rescue, Defense and Virulence				
PADG_01479	Gamma glutamyl transpeptidase	577,9	1,95	1
PADG_07460	Vacuolar aminopeptidase	693,28	2,34	1
PADG_06314	Carboxy peptidase Y	504,42	3,19	1
PADG_00634	Aspartyl protease	452,93	*	*
PADG_06992	Mitochondrial cochaperone GrpE	6890,69	2,39	1
PADG_07749	Protoplast secreted protein - Y20	38131,91	1,55	1
PADG_05183	Mitochondrial monothiol glutaredoxin 5	1304,22	J774	*
PADG_02764	Thioredoxin-likeprotein	2118,45	2,86	1
PADG_03161	Thioredoxin	647,52	*	*
PADG_05504	Mitochondrial thioredoxin	46080,04	1,22	1
PADG_03163	Mitochondrial cytochrome c peroxidase	6455,72	1,68	1
PADG_01954	Mitochondrial Fe/Mn superoxide dismutase	1207,88	1,42	1
PADG_07418	Cu/Zn superoxide dismutase	6827,38	1,77	1
PADG_00430	Mitochondrial HSP SSC1	25098,13	1,52	1
PADG_04984	MitochondrialHSP 10	99417,27	2,36	1
PADG_03963	HSP 30	3899,89	1,32	1
PADG_08369	HSP 60	63687,71	2,86	1
PADG_03562	Mitochondrial HSP70 protein	3219	1,40	1
PADG_05081	Aldehyde dehydrogenase	5771,94	1,22	1

Accession number[1]	**Protein description**	**Score**	**Fold change[2]**	**p value[3]**
Unclassified				
PADG_07627	4 carboxy muconolactone decarboxylase family protein	614,44	*	*
PADG_04439	Mitochondrial ATPase inhibitor	16899,15	3,67	1
PADG_04311	Zincknuckle domain-containing protein	1613,09	1,23	0,97
PADG_02652	RNP domain-containing protein	12768,48	1,36	1
PADG_06190	CCR4 Not complex subunit Caf16	571,48	*	*
PADG_03654	Conserved hypothetical protein	966,29	*	*
PADG_06087	dsDNA-binding protein PDCD5	1389,93	*	*
PADG_07923	Hypothetical protein	4253,59	1,34	1
PADG_03115	Hypothetical protein	2667,55	2,10	1
PADG_00944	Methyl transferase domain containing protein	324,84	*	*
PADG_03886	Conserved hypothetical protein	746,11	*	*
PADG_08724	RPEL repeat protein	594,42	*	*
PADG_01010	Predicted protein	1333,03	*	*
PADG_05584	Hypothetical protein	1281,44	*	*
PADG_05703	Hypothetica lprotein	6556,04	*	*
PADG_05739	NAP family protein	615,85	*	*
PADG_08736	Conserved hypothetical protein	509,84	*	*

[1] Accession number obtained in the *Paracoccidioides* database available a thttp://www.broadinstitute.org /annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] Fold change of the protein expression ratios obtained from *P. brasiliensis* yeast cells during macrophage infection : *Paracoccidioides* control condition**.** Proteins with fold change of 20% were considered regulated in *P. brasiliensis* during macrophage infection.

[3] *p*-value of the fold change. It was
considered statistically significant the p-values less than 0,05 and higher than 0,95.

* Proteins detected in*Pb*18 only during macrophage infection.

Supplementary table 2– Down-regulated proteins of *P. brasiliensis* during macrophage infection in J774 cells.

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Aminoacids metabolism				
PADG_00386	phospho 2 dehydro 3 deoxyheptonate aldolase	358,92	*	*
PADG_04698	aromatic ring-opening dioxygenase LigB subunit	325,63	*	*
PADG_05827	arginine biosynthesis bifunctional protein argJ	476,16	*	*
PADG_03149	Aminopeptidase	627,32	*	*
PADG_08304	acetolactate synthase small subunit	2250,36	*	*
PADG_02719	NAD dependent epimerase dehydratase family protein	441,59	0,52	0
PADG_08262	asparagine synthetase	556,4	0,64	0
PADG_00663	homoserine dehydrogenase	1112,96	0,70	0
PADG_05111	serine hydroxymethyl transferase	4952,93	0,79	0
PADG_03825	NAD specific glutamate dehydrogenase	1963,79	*	*
PADG_01305	arginino succinate lyase	28,45	*	*
PADG_00888	argininosuccinate synthase	3230,81	0,66	0
PADG_01404	aspartate amino transferase	1681,94	*	*
PADG_01928	S adenosylmethionine synthetase	6141,33	0,80	0
PADG_01615	homocitrate synthase	2480,23	*	*
PADG_06289	lysine decarboxylase-like protein	3085,26	*	*
PADG_00875	pentafunctional AROM polypeptide	534,7	*	*
PADG_05058	chorismate mutase	1627,34	*	*
PADG_05896	D 3 phosphoglycerate dehydrogenase	740,06	*	*
PADG_05301	cystathionine beta synthase	683,38	*	*
PADG_00349	kynureninase	607,61	*	*
PADG_06756	histidine biosynthesis trifunctional protein	938,05	*	*
PADG_08604	ATP phosphoribosyl transferase	1222,73	*	*
PADG_03167	3 isopropylmalate dehydratase	540,24	*	*
Nitrogen and sulfur metabolism				
PADG_00446	oxidoreductase 2 nitropropane dioxygenase family	3313,83	*	*
PADG_08569	thiosulfate sulfurtransferase	744,46	0,60	0
Nucleotide metabolism				
PADG_00209	guanine deaminase	321,64	*	*
PADG_00322	xanthine phosphoribosyltransferase 1	4746,3	*	*
PADG_01653	ribose phosphate pyrophosphokinase	675,7	*	*
PADG_05747	Phosphoribosyl formyl glycinamidine synthase	457,03	*	*
PADG_06297	phosphoribosylamine glycine ligase	617,21	*	*
PADG_07780	Phosphoribosyl glycinamide formyltransferase	601,88	*	*
PADG_04099	bifunctional purine biosynthesis protein ADE17	9141,72	0,55	0
PADG_07585	inosine 5 monophosphate dehydrogenase IMD2	2588,95	0,57	0
PADG_01100	uracil phosphoribosyl transferase	1640,11	*	*
PADG_05321	mitochondrial nuclease	1014,33	*	*
PADG_00780	ribose phosphate pyrophosphokinase	1879,31	*	*
C-compounds metabolism				
PADG_03118	Aldose 1-epimerase family protein	862,54	0,53	0
PADG_03943	Phospho mannomutase	1212,41	*	*
PADG_08386	Glycosyl transferase	541,65	*	*
PADG_01372	mannitol 1 phosphate 5 dehydrogenase	3295,53	0,73	0
PADG_04312	UDP N acetylglucosamine pyrophosphorylase	1304,79	0,76	0
PADG_03984	glucosamine fructose 6 phosphate aminotransferase	1156,98	*	*
PADG_07435	sorbitol utilization protein SOU2	2634,62	0,59	0
PADG_03859	NADP dependent mannitol dehydrogenase	1434,23	0,80	0
PADG_02132	mannose 6 phosphate isomerase	583,58	*	*
PADG_04374	UTP glucose 1 phosphate uridylyltransferase	2352,22	*	*
PADG_01486	short chain dehydrogenase reductase family	623,71	*	*
PADG_03278	myo inositol 1 phosphate synthase	648,34	*	*

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Signal transduction				
PADG_01480	mitogen activated protein kinase MKC1	535,56	*	*
PADG_03544	ser Thr protein phosphatase family protein	618,15	*	*
PADG_08337	GTP binding protein rho1	4823,75	0,75	0
PADG_04056	14 3 3 protein epsilon	18566,56	0,81	0
PADG_04440	14 3 3 like protein 2	64823,43	0,81	0
PADG_02212	serine threonine protein phosphatase PPT1	1134,79	*	*
PADG_08399	GTP binding protein ypt3	3662,66	*	*
PADG_01849	GTP dependent nucleic acid binding protein engD	1100,01	0,75	0
PADG_01243	rab GDP dissociation inhibitor	6219,94	*	*
PADG_04810	GTP binding nuclear protein GSP1 Ran	19149,31	0,30	0
PADG_02800	adenylyl cyclase associated protein	472,62	*	*
Transcription and transcriptional control				
PADG_07689	transformer-SR ribonucleoprotein	595,58	*	*
PADG_06734	U6 snRNA binding protein	927,95	*	*
PADG_06768	rRNA 2 O methyltransferase fibrillarin	534,02	*	*
PADG_05034	RNA binding domain-containing protein	1736,7	*	*
PADG_06856	small nuclear ribonucleoprotein Sm D2 115 aa	528,54	*	*
PADG_03733	Poly rC binding protein	1011,45	*	*
PADG_02473	pirin	694,39	*	*
PADG_07134	histone H4	2253,29	*	*
PADG_07884	polyadenylate binding protein	1003,83	0,58	0
Mitotic cell cycle and cell cycle control				
PADG_00824	telomere and ribosome associated protein Stm1	1417,5	0,84	0
PADG_01796	caffeine-induced death protein Cid2	552,99	*	*
PADG_02763	cyclin dependent kinases regulatory subunit	3505,33	0,76	0
PADG_05893	nucleosome assembly protein	2676,57	*	*
PADG_05907	histone H2B type 1 A	2731,64	*	*
PADG_05931	condensin	506,43	*	*
PADG_05683	cell division control protein	1676,86	0,78	0
Glycolysis				
PADG_01896	phosphoglycerate kinase	10933,7	0,20	0
PADG_03813	hexokinase	1005,71	*	*
PADG_00192	6 phosphofructokinase	1158,94	*	*
PADG_01278	pyruvate kinase	13912,69	0,73	0
PADG_06906	triosephosphate isomerase	11571,56	0,79	0
PADG_00668	fructose bisphosphate aldolase	19990,67	0,37	0
Tricarboxilic acid				
PADG_02063	pyruvate dehydrogenase E1 component subunit alpha	1202,79	*	*
PADG_02805	isocitrate dehydrogenase subunit 2	1872,1	*	*
PADG_03977	isocitrate dehydrogenase subunit 1	3357,02	0,51	0
PADG_00317	succinyl CoA ligase subunit beta	1074,65	*	*
PADG_08387	citrate synthase	7729,15	0,36	0
PADG_01762	2 oxoglutarate dehydrogenase E1	718,56	0,77	0
Pentose phosphate pathway				
PADG_03651	6 phosphogluconate dehydrogenase	2292,53	0,79	0
Glyoxalate cycle				
PADG_04702	malate synthase	2960,63	*	*
Methylcitrate cycle				
PADG_04710	2 methylcitrate synthase	17679,08	0,28	0

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Electron transport and respiration				
PADG_07042	ATP synthase subunit 5	1549,49	*	*
PADG_06978	cytochrome c	7687,89	0,76	0
PADG_02578	ATP synthase subunit 4	596,35	*	*
PADG_02669	electron transfer flavoprotein subunit beta	2895,44	*	*
PADG_03872	NADH cytochrome b5 reductase	955,33	0,57	0
PADG_07081	electron transfer flavoprotein subunit alpha	1315,88	0,59	0
PADG_07964	vacuolar ATP synthase subunit E	1326,39	0,70	0
PADG_08394	cytochrome b c1 complex subunit 2	720,82	*	*
PADG_06956	vacuolar ATP synthase subunit B	705,81	0,82	0,02
Lipid, fatty acid and isoprenoid metabolism				
PADG_07508	phosphatidylinositol transfer protein SFH5	3076,09	0,76	0
PADG_00254	fatty acid synthase subunit alpha reductase	1110,48	*	*
PADG_00255	fatty acid synthase subunit beta dehydratase	2244,05	*	*
PADG_03449	isopentenyl diphosphate Delta isomerase	807,48	*	*
PADG_08651	peroxisomal multifunctional enzyme	1142	*	*
PADG_04495	4 coumarate CoA ligase	714,31	0,73	0
PADG_01228	3 hydroxybutyryl CoA dehydrogenase	4535,03	0,82	0
PADG_06876	3 hydroxyisobutyryl CoA hydrolase	3141,18	*	*
PADG_01687	3 ketoacyl CoA thiolase	5137,5	0,47	0
PADG_04994	ATP citrate lyase	1738,19	0,67	0
PADG_01677	acetyl coenzyme A synthetase	655,5	*	*
Biosynthesis of cofactor and vitamins				
PADG_00443	dihydropteroate synthase	1062,11	*	*
PADG_03983	6 7 dimethyl 8 ribityllumazine synthase	1169,47	0,71	0
PADG_01897	chlorocatechol 1 2 dioxygenase	857,03	*	*
PADG_03944	26S proteasome non ATPase regulatory subunit 10	27,87	*	*
Cell Growth and morphogenesis				
PADG_05538	actin	1639,2	*	*
PADG_07249	actin binding protein	1135,38	0,57	0
PADG_05341	fimbrin	604,95	*	*
PADG_00128	tubulin alpha chain	665,15	*	*
PADG_05491	GMF family protein, actin depolymerization factor	2939,5	0,66	0,03
PADG_03959	ARP2 3 actin organizing complex subunit Sop2	1031,25	0,63	0
PADG_07930	ARP2 3 complex 20 kDa subunit	1695,34	*	*
PADG_07733	ARP2 3 complex 34 kDa subunit	661,72	*	*
Protein synthesis				
PADG_07888	eukaryotic translation initiation factor 5A	4811,73	0,83	0,03
PADG_04057	translation initiation factor eIF3	4000,27	*	*
PADG_00932	translation initiation factor eIF3	478,38	*	*
PADG_01891	translation initiation factor RLI1	656,19	*	*
PADG_06110	translation factor SUI1	3845,67	*	*
PADG_00692	elongation factor 1 alpha	25477,84	0,23	0
PADG_02759	ribosome recycling factor domain-containing protein	1902,46	*	*
PADG_02752	116 kDa U5 small nuclear ribonucleoprotein component	110,37	0,53	0
PADG_04307	mRNA binding post transcriptional regulator Csx1	2870,54	0,71	0
PADG_04730	nascent polypeptide associated complex subunit alpha	1887,05	*	*
PADG_02896	elongation factor 1 beta	27699,38	0,36	0
PADG_06265	elongation factor 1 gamma 1	21636,54	0,41	0
PADG_08125	elongation factor 2	8384,21	0,45	0
PADG_03431	putative tRNA-binding protein	533,64	*	*
PADG_03440	prolyl tRNA synthetase	454,75	*	*
PADG_01558	histidyl tRNA synthetase	1005,73	*	*
PADG_02484	valyl tRNA synthetase	844,82	*	*
PADG_03689	tyrosyl tRNA synthetase	1451,04	*	*
PADG_05848	glycyl tRNA synthetase	600,14	*	*
PADG_05897	seryl tRNA synthetase	2707,68	*	*

PADG_08472	lysyl tRNA synthetase	857,74	*	*
PADG_04962	aspartyl tRNA synthetase	2972,06	0,54	0
PADG_00785	ribosomal protein S15	1042,95	*	*
PADG_07583	ribosomal protein S21E	11069,39	0,84	0
PADG_01654	ribosomal protein S6 PA	6916,45	0,74	0
PADG_02142	ribosomal protein L5	13042,87	0,75	0
PADG_04449	ribosomal protein L23e	2224,35	0,78	0
PADG_01267	30S ribosomal protein S17P	4145,94	0,72	0
PADG_01503	37S ribosomal protein Rsm24	553,96	*	*
PADG_03315	40S ribosomal protein S4	5778,31	0,72	0
PADG_06838	40S ribosomal protein S5	8692,07	0,84	0
PADG_07863	40S ribosomal protein S8	7473,32	0,77	0
PADG_03326	40S ribosomal protein S9	8370,36	0,71	0
PADG_04866	40S ribosomal protein S10 A	3053,9	*	*
PADG_01427	40S ribosomal protein S12	3182,33	0,69	0
PADG_07685	40S ribosomal protein S13 1	8064,19	0,78	0
PADG_02445	40S ribosomal protein S15	10623,49	0,39	0
PADG_00333	40S ribosomal protein S16	4491,59	0,76	0
PADG_00354	40S ribosomal protein S17	12836,03	0,79	0
PADG_06502	40S ribosomal protein S20	7199,8	0,48	0
PADG_06680	40S ribosomal protein S22	4014,56	0,83	0
PADG_06599	40S ribosomal protein S25	539,48	*	*
PADG_08605	40S ribosomal protein S28	4991,9	*	*
PADG_00784	40S ribosomal protein S0	8686,2	0,82	0
PADG_04030	60S acidic ribosomal protein P0	7617,59	0,68	0
PADG_02249	60S ribosomal protein L2	7260,73	0,70	0
PADG_07173	60S ribosomal protein L3	2294,24	0,77	0
PADG_05721	60S ribosomal protein L4 A	4738,24	0,70	0
PADG_01387	60S ribosomal protein L7	2449,69	0,68	0
PADG_04848	60S ribosomal protein L8 B	14038,4	0,63	0
PADG_02828	60S ribosomal protein L10a	1216,32	0,59	0
PADG_03778	60S ribosomal protein L10	3824,79	0,68	0
PADG_07803	60S ribosomal protein L12	9790,37	0,63	0
PADG_03856	60S ribosomal protein L15	8385,96	0,73	0
PADG_00514	60S ribosomal protein L16	3425,75	0,73	0
PADG_06726	60S ribosomal protein L17	2919,98	0,66	0
PADG_05264	60S ribosomal protein L19	4454,72	0,68	0
PADG_03873	60S ribosomal protein L20	4368,79	0,70	0
PADG_03325	60S ribosomal protein L21 A	9303,96	0,77	0
PADG_05883	60S ribosomal protein L23a	11162,32	0,82	0
PADG_00612	60S ribosomal protein L27 A	8874,16	0,69	0
PADG_05939	60S ribosomal protein L27a	489,74	0,78	0,02
PADG_03781	60S ribosomal protein L30	10975,43	0,83	0
PADG_01083	60S ribosomal protein L32	6304,8	0,78	0
PADG_01026	60S ribosomal protein L43	7369,92	0,66	0
Protein folding				
PADG_00050	T complex protein 1 subunit alpha	1353,14	*	*
PADG_00928	T complex protein 1 subunit gamma	1340,39	*	*
PADG_02057	T complex protein 1 subunit theta	1461,5	*	*
PADG_03441	T complex protein 1 subunit alpha	604,66	*	*
PADG_08048	T complex protein 1 subunit beta	981,88	*	*
PADG_08234	T complex protein 1 subunit eta	444,18	*	*
PADG_08484	T complex protein 1 subunit epsilon	827,58	*	*
PADG_06488	peptidyl prolyl cis trans isomerase D	20036,47	0,68	0
PADG_00001	peptidyl prolyl cis trans isomerase H	11825,86	0,82	0
Protein targeting, sorting and translocation				
PADG_04034	mitochondrial protein import protein MAS5	441,38	*	*
PADG_00187	SNARE Ykt6	739,12	*	*
PADG_00326	ADP ribosylation factor	638,49	*	*
PADG_02833	ADP ribosylation factor	6678,63	0,73	0

Accession number[1]	Protein description	Score	Fold change[2]	p value[3]
Protein modification and assembly				
PADG_00602	HNRNP arginine N methyltransferase	874,79	*	*
Protein degradation				
PADG_06851	26S proteasome non ATPase regulatory subunit 11	693,35	*	*
PADG_00599	26S protease regulatory subunit 6A	946,17	*	*
PADG_00631	proteasome 26S subunit	2324,02	*	*
PADG_03192	proteasome subunit alpha type 5 A	1071,37	*	*
PADG_04067	proteasome component PUP3	3288,96	0,61	0
PADG_02637	ubiquitin conjugating enzyme	1449,18	0,69	0
PADG_03424	ubiquitin-activating enzyme E1	737,43	0,66	0
PADG_04167	aspartyl aminopeptidase	888,45	*	*
PADG_08265	methionine aminopeptidase	680,33	*	*
Transport				
PADG_06033	NIPSNAP family protein	3382,2	0,33	0
PADG_08176	phosphatidylinositol phosphatidylcholine transfer protein	1546,75	*	*
PADG_06378	carnitine acyl carnitine carrier	486,8	*	*
PADG_08368	Cofilin	4118,88	0,33	0
PADG_00622	arsenical pump driving ATPase	1226,15	*	*
PADG_01847	stomatin family protein	582,14	*	*
PADG_01440	ADP ATP carrier protein	1281,41	*	*
Cell Rescue, Defense and Virulence				
PADG_07674	carbonic anhydrase	1294,52	0,64	0
PADG_00324	peroxisomal catalase	7532,22	0,54	0
PADG_04587	peroxiredoxin HYR1	424,23	*	*
PADG_04912	AhpC/TSA-thioredoxin peroxidase/alkyl hydroperoxide reductase	5662	0,61	0
PADG_07946	peroxisomal matrix protein	6803,02	0,63	0
PADG_02030	Hsp90 co chaperone Cdc37	1582,78	*	*
PADG_00765	heat shock protein HSP98	987,2	*	*
PADG_05139	heat shock 70 kd protein cognate 1	995,3	0,67	0
PADG_03927	DNAJ domain containing protein	691,37	*	*
PADG_08118	hsp70 like protein	62668,96	0,66	0
PADG_07715	heat shock protein	10535,23	0,50	0
PADG_02761	heat shock protein SSB1	1560,43	*	*
PADG_04379	heat shock protein STI1	3476,82	0,75	0
PADG_01711	Hsp90 co chaperone AHA1	2746,85	0,54	0
Unclassified				
PADG_06709	zinc binding oxireductase domain containing protein	424,27	*	*
PADG_00541	hypothetical protein	1679,04	*	*
PADG_03031	CobW domain containing protein	1372,54	*	*
PADG_03121	DUF757 domain containing protein	583,55	*	*
PADG_03203	BAR domain protein	751,09	*	*
PADG_03244	HD domain containing protein	1493,64	*	*
PADG_07355	conserved hypothetical protein	27,87	*	*
PADG_07412	DUF1479 domain-containing protein	897,69	*	*
PADG_07670	SAP domain protein	689,63	*	*
PADG_08480	conserved hypothetical protein	585,72	*	*
PADG_05701	conserved hypothetical protein	338,45	0,02	0
PADG_01488	thiol methyltransferase	11310,88	0,68	0
PADG_04636	dienelactone hydrolase family protein	4043,96	0,82	0

[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] Fold change of the protein expression ratios obtained fromyeast cells during macrophage infection : yeast cells fromcontrol condition**.** Proteins with fold change of 20% were considered regulated in *P. brasiliensis* during macrophage infection.

[3] *p*-value of the fold change. It was considered statistically significant the p-values less than 0,05 and higher than 0,95.

* Proteins detected in *Pb*18 only during control condition.

5 DISCUSSÃO

O estudo de interações entre o fungo e o hospedeiro constitui uma importante estratégia na avaliação da patogênese de *Paracoccidioides spp.* As análises proteômicas das respostas de *Paracoccidioides Pb*18 durante a infecção foram realizadas pela primeira vez neste trabalho. Após padronização, um protocolo preciso, útil na obtenção de leveduras recuperadas após a interação, foi estabelecido e ajudará futuros estudos proteômicos visto que somente proteínas de *Paracoccidioides* e não de macrófagos foram identificadas por espectrometria de massas.

No intuito de estudar o processo de infecção e a interação fungo-hospedeiro, realizamos a análise do perfil proteômico de células leveduriformes de *Paracoccidioides Pb18* recuperadas da infecção em macrófagos J774 A1. Os extratos proteicos obtidos foram analisados por Cromatografia Líquida associada à espectrometria de massas Nano UPLC-MS^E. A análise identificou um total de 533 proteínas em condições de infecção com *Pb* 18 em macrófagos J774 A1.

Nas análises comparativas de dados visando à detecção de expressão diferencial de proteínas, foi possível detectar 181 proteínas com indução da expressão e a diminuição da expressão foi observada em 245 proteínas no isolado *Pb*18. As proteínas foram identificadas e classificadas em categorias funcionais.

Muitos estudos de resposta e adaptação do patógeno baseiam-se no fato de que estes patógenos estão expostos à variações ambientais como temperatura, estresse oxidativo e limitação de nutrientes e visam mimetizar as condições encontradas pelo patógeno *"in vivo"* (Tavares *et al.*, 2007, Rai *et al.*, 2012).

As análises proteômicas de interação do fungo *Paracoccdiodides Pb*18 com macrófagos J774 A1 revelam a presença de proteínas relacionadas à vários processos fornecendo uma visão global da resposta do fungo durante a infecção. Os dados obtidos sugerem a adaptação e versatilidade do fungo ao ambiente hostil do macrófago buscando a sobrevivência. As vias mais relevantes relacionados à esta condição assim como as proteínas identificadas são discutidas abaixo.

5.1 Metabolismo e Energia

As alterações metabólicas detectadas nos níveis de expressão de proteínas revelam como células leveduriformes de *Paracoccidioides* respondem ao meio do macrófago. Como já descrito em fungos como *C.albicans*, genes relacionados ao ciclo do glioxilato são induzidos após exposição à macrófagos e neutrófilos (Lorenz *et al*., 2004) o que facilitaria a assimilação de compostos com dois carbonos. O processo de ativação do ciclo do glioxilato em resposta à fagocitose é importante pois permite a adaptação de patógenos intracelulares ao microambiente pobre em glicose dos macrófagos permitindo a assimilação de fontes simples como o acetato ou ácidos graxos para gliconeogênese dentro de fagossomos (Lorenz & Fink, 2002). Observamos a indução da (isocitrate lyase), enzima chave do ciclo do glioxilato, nos dois isolados enquanto a enzima malato sintase (malate synthase) apresentou-se reprimida no isolado *Pb*18.

Como já foi descrito, o fagossomo apresenta um meio pobre em glicose e aminoácidos, o que requer a adaptação de patógenos como fungos e bactérias (Lorenz *et al.*, 2004). A resposta proteômica de *Paracoccidioides* mostrou uma clara diminuição da expressão de proteínas relacionadas à via glicolítica indicando que o meio é deficiente em glicose como mostrado nas enzimas relacionadas com a glicólise, incluindo as enzimas reguladoras hexoquinase e piruvato-quinase. A ativação da gliconeogênese foi mostrada pela expressão induzida das enzimas fosfoenol piruvato quinase (phosphoenol piruvate kinase) no *Pb*01 e frutose 1,6 bisfosfatase (fructose 16 bisphosphatase) no *Pb*18. Avaliando o metabolismo anaeróbico, observamos a regulação positiva das enzimas relacionadas à fermentação alcoólica como álcool desidrogenase (álcohol dehydrogenase) nos dois isolados e piruvato-decarboxilase (pyruvate decarboxylase) em *Pb*18 sugerindo a produção de etanol aumentada.Também é de suma importância notar que a utilização do glicogênio esteja aumentada em células de leveduras de *Paracoccidioides Pb*18 durante fagocitose uma vez que as enzimas glicogênio-fosforilase (glycogen phosphorilase) e fosfoglicerato-mutase (phosphoglicerate mutase) são positivamente reguladas induzindo ao catabolismo.

Durante o processo de infecção, ocorre a liberação de lipídeos e o crescimento de patógenos em substratos como ácidos graxos requerem o ciclo de beta oxidação catabólico e ciclo do glioxilato e o propionol coA gerado do processo de beta oxidação que é metabolizado via ciclo do metil citrato (Muñoz-Elias *et al*., 2006, Dubey *et al*., 2013). A beta oxidação de ácidos graxos pode produzir acetil coA e propionil coA, que também pode ser produzido a partir de aminoácidos de cadeia ramificada. Nós observamos o aumento da indução de enoil coA hidratase (enoyl co A hydratase) nos dois isolados. Em fungos e bactérias, o propionil coA é assimilado via ciclo do metil citrato, o qual converte propionil coA em piruvato (Bramer *et al*., 2002). Três enzimas são específicas do ciclo do metil citrado: metil citrato desidratase MCD, metilcitrato sintase MCS e metilisocitrato liase MCL. Nos isolados *Pb*01 e *Pb*18 foi observada a diminuição na expressão de MCS, sendo que MCD foi aumentada nos dois isolados em *Pb*01 e MCL em *Pb*18.

A indução de proteínas relacionadas à beta oxidação de ácidos graxos, ciclo do ácido cítrico e transporte de elétrons sugerem a utilização como fontes de energia. A gliconeogênese também induzida sugere a conversão de piruvato para a produção de glicose importante na resposta adaptativa à fagocitose.

5.2 Transcrição, Síntese e Ciclo Celular

Estudos tem mostrado a regulação negativa dos processos envolvidos com o crescimento celular, isto é, relacionados com os vários aspectos do metabolismo de RNA, por exemplo, síntese e processamento de tRNA, iniciação e elongação da tradução e processamento e *splicing* de RNA, bem como de genes codificantes para proteínas ribossomais. A síntese de proteínas é regulada durante o processo de infecção como mostrada em várias proteínas, tais como fatores de iniciação da tradução, tRNAsynthetases aminados e proteínas ribossomais. A diminuição na expressão de proteínas ribossomais foi observada nos dois isolados e de proteínas relacionadas aos processos de síntese, modificações e processamento de proteínas.

Nós observamos a indução nos dois isolados na expressão de proteínas relacionadas ao proteassoma que representa um complexo necessário para degradação e eliminação de proteínas tóxicas e não funcionais em função dos diversos processos envolvidos durante a infecção prevenindo o acúmulo de proteínas. A indução na expressão de proteínas relacionadas ao metabolismo de aminoácidos sugere que *Pb01 e Pb18* aumentaram expressão de enzimas relacionadas à síntese de aminoácidos que participam da gliconeogênese (glicogênicos) como glicina, metionina, glutamato, isoleucina. Em *Pb18* as enzimas relacionadas ao glutamato (desidrogenase específica- glutamatedehydrogenase, alanina (aminotransferase glioxilato alanina-alanineglyoxylateaminotransferase) e aspartatato (sintetase adenil succinato-adenylosuccinatesynthetase) foram induzidas em *Pb*18 resultando no enriquecimento de precursores de glicose como piruvato, fumarato e oxaloacetato. Houve também indução das enzimas relacionadas ao catabolismo de aminoácidos sugerindo a possibilidade de reciclagem de moléculas na produção de energia.

5.3 Estresse, Defesa e Virulência

Diferentes grupos funcionais como o estresse oxidativo, detoxificação, reparo ao DNA e patogênese são inclusos nesta avaliação. Os patógenos quando fagocitados são submetidos à moléculas antimicrobianas como espécies reativas de oxigênio (EROS) gerados pelo complexo NADP oxidase e iNOS (Missal *et al.,* 2004) e que necessitam de detoxificação. As estratégias usadas pelo fungo para escapar destas moléculas incluem a detoxificação enzimática de espécies reativas, resposta ao estresse e reparo do DNA.

O nosso estudo sobre o perfil proteômico de *Paracoccidioides* revelou que este fungo estimula respostas moleculares para resistir aos danos causados por meio da produção de várias enzimas antioxidantes que atuam no processo de detoxificação (Grossklaus *et al*., 2013, Silva *et al*., 2008, Ruiz *et al*., 2011). As enzimas que compõem a defesa antioxidante incluem catalases (Hollbrock et al., 2013) super óxido dismutase SOD (Zelko *et al*., 2002), citocromo c peroxidase (CPP), tioredoxina, proteínas de choque térmico dentre outras.

A enzima super óxido dismutase (SOD-super oxide dismutase) tem sido descrita como um fator de virulência em fungos e bactérias pois realizam a detoxificação de moléculas EROS ânion superóxido e peróxido de hidrogênio (Hamilton *et al.,* 1999). Assim também observamos no nosso estudo no isolado *Pb*18, a identificação de duas super óxido dismutases SOD Ferro/Manganês (Fe/Mn) e SOD Cobre/Zinco(Cu/Zn) induzidas possivelmente em resposta ao estresse oxidativo no interior dos macrófagos.

A indução de peroxidases revela a resposta antioxidante de *Paracoccidioides* durante a infecção. A enzima citocromo C peroxidase utiliza o peróxido como um aceptor de elétrons para oxidar o citocromo C no espaço intermembranar da mitocôndria e em *Paracoccidioides* foi verificado que a citocromo C peroxidase pode auxiliar na resistência ao estresse oxidativo por meio da detoxificação dos elevados níveis de H_2O_2 (Dantas *et al*., 2008). Foi evidenciada a indução de citocromo C peroxidase **(**cytochrome c peroxidase) observada nos dois isolados. Uma de nossas estratégias para avaliação do perfil proteômico de *Paracoccidioides* em resposta ao estresse no meio do macrófago foi avaliar a resposta do mutante de *Paracoccidioides* para citocromo c peroxidase-CPP que mostrou a importante atuação desta enzima durante o processo de infecção.

O sistema tioredoxina (thioredoxin**)** é composto por diversas moléculas que interagem com resíduos de cisteína, sendo um dos principais reguladores no processo de redução de tióis na célula. A produção de EROs modula o estado tiol-redox celular e neste contexto, o sistema tioredoxina torna-se muito importante, uma vez que pode restaurar o balanço entre redução e oxidação celular, favorecendo assim o funcionamento normal da célula em condições de estresse (Yoshihara *et al*., 2010). Em *S. cerevisiae* observou-se que a super expressão do gene codificante para a tioredoxina aumenta a defesa redox (Gomez-Pastor *et al*., 2010) e que o sistema tioredoxina pode funcionar como uma alternativa na redução da glutationa oxidada *in vivo*, o que aumentaria a resistência destas leveduras ao estresse oxidativo (Tan *et al*., 2010). O aumento na expressão do gene tioredoxina foi observado nos dois isolados o que pode indicar um estímulo para o balanço redox.

A enzima gama glutamil transpetidase (gamma-glutamyltranspeptidase), tem sido descrita como fator de ativação de cascatas de estresse oxidativo e foi identificada com níveis aumentados em *Pb*18. É importante ressaltar a super expressão de proteínas relacionadas à processo de degradação autofágica de proteínas observadas em *Pb*18, tais como aminopeptidase vacuolar, carboxipeptidase Y e aspartil protease. A indução de degradação de proteínas gera aminoácidos frente à pouca disponibilidade de nutrientes no fagolisossomo.

O estresse oxidativo é uma condição onde pode ser observada a indução de proteínas de choque térmico (HSP-Heat Shock Proteins). As proteínas de choque térmico apresentam diversas funções em processos celulares como a divisão celular, síntese de DNA, transcrição, tradução, enovelamento e transporte (Liberek et al., 2008) auxiliando na proteção celular e m processos causados pelo estresse. As chaperonas são exemplos de moléculas antioxidantes secundárias que possivelmente atuaram no reparo aos danos causados pelo peróxido e também desempenham papel como chaperonas moleculares em condições normais ou de estresse. Elas facilitam o processamento de peptídeos nascentes (Schelesinger *et al.,* 1990), ajudando no transporte molecular e protegem as células da apoptose induzidas por óxido nítrico, Fator de Necrose Tumoral (TNF), hipóxia e condições de estresse (Samamli *et al*., 1998) restaurando o enovelamento de proteínas crítico em situações de estresse (Brosnan *et al*., 2000). A proteína de choque térmico 60 (Hsp 60) é uma das proteínas descritas com aumento na expressão em análise diferencial quando da interação de macrófagos RAW 264.7 com *C.albicans* (Martinez-Solano *et al.,* 2006). Níveis aumentados para a proteína de choque térmico 30 (HSP 30) foram observados em *Pb01,* já descrita em processo infeccioso; proteína de choque térmico 10 (HSP 10) para o isolado *Pb*01 e proteína de choque térmico 70 (HSP 70) para o isolado *Pb*18.

A infecção por *Paracoccidioides spp* está relacionada à sua capacidade adaptativa ao ambiente do hospedeiro, ressaltando que a mitocôndria assume papel de relevância na adaptação metabólica a estresses ambientais. A cadeia respiratória mitocondrial é ramificada em cadeia clássica dos citocromos e cadeia alternativa da enzima oxidase alternativa. As enzimas relacionadas à este processo apresentaram indução nos dois isolados.

A identificação de fatores de virulência em fungos e reconhecimento de mecanismos de patogênese devem ajudar no desenvolvimento de novas terapias antifúngicas. Os fatores de virulência em fungos patogênicos humanos são descritos como aderência, secreção de enzimas hidrolíticas e dimorfismo em *C.albicans*; habilidade de crescimento à temperatura de 37° C, síntese de cápsula e formação de melanina em *C. neoformans*; produção de pigmento, moléculas de adesão e secreção de enzimas hidrolíticas em *A. fumigatus (*Kuleta *et al*., 2009). As proteínas Gliceraldeído-3-fosfato desidrogenase (glyceraldehyde 3 phosphate dehydrogenase-GAPDH) (Barbosa *et al*., 2006) e enolase (enolase) (Nogueira *et al*., 2010) foram identificadas em *Pb*18 e *Pb*01 com regulação positiva, sugerindo papel de adesão do micro-organismo aos tecidos do hospedeiro no processo de infecção.

5.4 Comunicação Celular e Mecanismo de Transdução de Sinais

Em condições de infecção com o isolado *Paracoccidioides Pb*18 observamos a indução de proteínas relacionadas à sinalização celular como calcineurina subunidade B (calcineurin subunit B), calmodulina (calmodulin), proteína de ligação ao GTP SAS 1 (GTP binding protein SAS 1), proteína da família fosforilase (phosphorylase family protein) e proteína partícula reconhecedora de sinais (signal recognition particle protein). Os níveis de expressão da proteína calmodulina também estão aumentados em *Paracoccidioides Pb*01.

As moléculas que induzem o estresse oxidativo no interior dos macrófagos produzem sinais que podem ser reconhecidos por receptores na superfície do fungo como a proteína SAS1 (proteína de ligação ao GTP SAS 1). Esta proteína está envolvida na sinalização intracelular e associada com função na membrana e ligação ao GTP. Após o reconhecimento e interação entre receptores e ligantes auxiliado por proteína que contém partícula reconhecedora de sinal (proteína partícula reconhecedora de sinais), as moléculas envolvidas nesta interação enfatizam as relações com a ativação de vias de sinalização. A ligação ligante-receptor leva ao aumento do cálcio citosólico, o que estimula a ligação à calmodulina e pode ativar os mecanismos de transcrição. A proteína calmodulina liga-se ao cálcio e modifica sua interação com outras proteínas, muitas proteínas que ligam-se à calmodulina não são capazes de ligar-se ao cálcio e levar à modificações. O cálcio participa como segundo mensageiro podendo levar à ativação de fatores de transcrição, como proteínas reguladoras. Os mecanismos de sinalização são utilizados pelo fungo para controlar a expressão de genes envolvidos na resposta ao estresse oxidativo. Muitas vias de sinalização são reguladas por fosforilação (proteína da família fosforilase) e fosfatases, que promovem a defosforilação via calcineurina controlada pelo cálcio celular (**calcineurina subunidade B**).

O estresse oxidativo presente no fagolisossomo ativa a resposta do fungo via sinalização celular com reprogramação da expressão gênica induzindo a expressão de genes necessários para conter a elevação letal de ROS com função antioxidante pra restaurar o potencial redox celular. A indução das proteínas citadas reflete a ativação da via Ca/calmodulina/calcineurina, uma das vias de sinalização descritas em *Paracocidioides* que controla a sobrevivência em condições de estresse oxidativo.

5.5 Considerações Finais

Os resultados obtidos fornecem evidências da importância das vias metabólicas no fungo *Paracoccidioides Pb*01 e *Pb*18 como metabolismo e energia, síntese e destino das proteínas, defesa e virulência, comunicação celular, o que nos leva a descrever a resposta adaptativa do fungo durante o processo de infecção em macrófagos. Em função do ambiente do fagolisossomo, o fungo apresenta a ativação de enzimas antioxidantes e um rearranjo do metabolismo observados por ativação de vias para minimizar os efeitos oxidativos.

A reprogramação do metabolismo de carbono caracterizada pela diminuição da glicólise e aumento da gliconeogênese, ciclo do glioxilato e degradação de ácidos graxos com indução de metabolismo de lipídeos (Lorenz *et al.*, 2004), defesa e virulência, detoxificação, reparo e resposta ao estresse (Fernandez-Arenas *et al.*, 2007, Brock *et al.*, 2009) são características de fungos patogênicos internalizados em macrófagos que definem a adaptação metabólica às condições de infecção (Brown *et al.,* 2007). Os resultados proteômicos obtidos durante os estudos de interação refletem a adaptação do patógeno ao nicho estimulando o sistema de defesa e detoxificação associada com alterações no metabolismo como: inibição da expressão de proteínas relacionadas à glicólise e aumento na expressão de proteínas durante a gliconeogênese ressaltando que os precursores de glicose são obtidos a partir do catabolismo de aminoácidos também induzido, beta oxidação de ácidos graxos e ciclo do glioxilato (Costa *et al*., 2007), bem como a inibição da síntese de proteínas visando à obtenção de nutrientes. A resposta ao estresse oxidativo é estimulada pela indução de enzimas antioxidantes como a citocromo c peroxidase, importante para a sobrevivência de *Paracoccidioides spp* no processo de infecção, como mostrado em experimentos de linhagens mutantes.

A identificação de proteínas expressas por *Paracoccidioides Pb*01 e *Pb*18 durante a infecção em macrófagos revelou o envolvimento de proteínas em processos metabólicos essenciais à sobrevivência e adaptação do fungo ao meio ambiente do hospedeiro. O perfil proteômico de *Paracoccididioides Pb 18* em resposta à internalização em macrófagos descrito sugere significativo remodelamento do metabolismo frente ao estresse oxidativo, limitação de nutrientes que leva ao aperfeiçoamento do sistema bioquímico na captação de nutrientes e ressalta o desenvolvimento de novas estratégias de sobrevivência do fungo. Espera-se que novas identificações de proteínas possam ser feitas para que possamos entender o perfil de infecção com *Pb*01 e condições de virulência. Ainda o conhecimento sobre as exigências metabólicas do fungo patogênico determinadas pelo nicho do hospedeiro durante a infecção, pode levar à identificação de novas classes de antifúngicos. Além disso, o trabalho abre espaço para futuros estudos de proteômica na infecção em outros fungos visto que, através deste protocolo, proteínas de *Paracoccidioides Pb01 e Pb18* durante o processo infeccioso foram identificadas.

6 CONCLUSÕES

O perfil proteômico de *Paracoccidioides sp* em resposta à infecção em macrófagos foi caracterizado pela primeira vez em nosso estudo. Utilizando como estratégia experimental a proteômica, foi possível identificar com êxito proteínas expressas em condições que mimetizam a infecção no hospedeiro. A caracterização das proteínas diferencialmente expressas identificadas por espectrometria de massas através das análises comparativas de dados possibilitou a detecção:

Com indução na expressão: 181 proteínas *Pb*18
Com diminuição na expressão: 245 proteínas em *Pb*18,

A análise proteômica nos possibilitou estudar os eventos moleculares associados à resposta adaptativa de *Paracoccidioides sp* ao meio do macrófago durante a infecção. O estresse oxidativo presente após internalização do fungo sugere a ativação das enzimas antioxidantes capazes de detoxificar e minimizar os efeitos causados. As alterações presentes sugerem o remodelamento do metabolismo do fungo observado por alterações como a ativação de enzimas da gliconeogênese e do catabolismo de aminoácidos sugerindo a utilização de diferentes estratégias para sobrevivência abrindo espaço para futuros estudos da interação patógeno-hospedeiro.

7 PERSPECTIVAS

1. Validação da expressão das proteínas de interesse selecionadas (diferencialmente expressas) e os genes correspondentes analisados pelo conteúdo dos transcritos por RT - PCR em tempo real.
2. Avaliação das vias metabólicas induzidas e reprimidas durante o processo infeccioso
3. Avaliação do perfil de expressão de proteínas durante a infecção em outros isolados de *Paracoccidioides spp*

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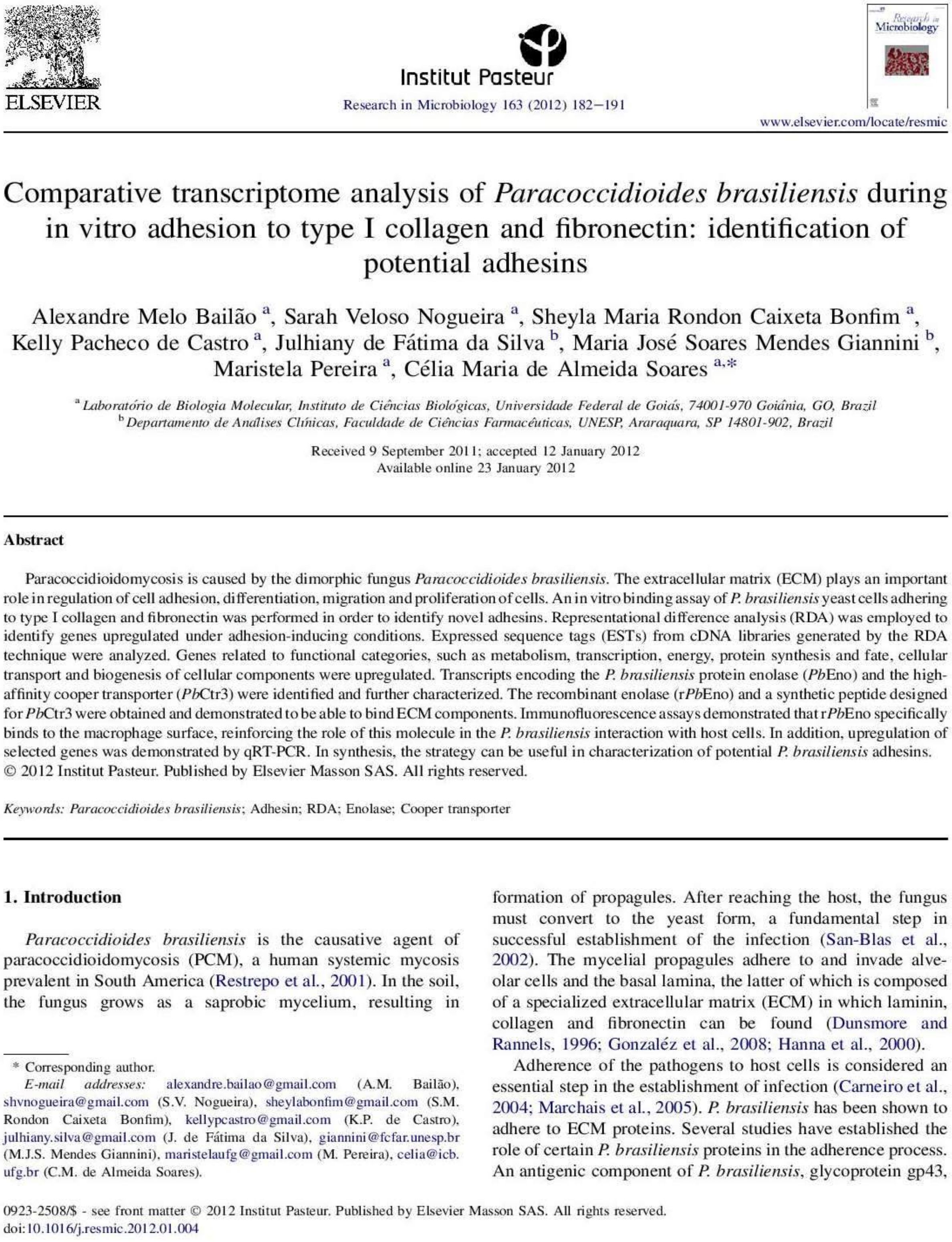
ANEXOS

Anexo 1 – Artigo completo publicado em periódico



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Comparative transcriptome analysis of *Paracoccidioides brasiliensis* during in vitro adhesion to type I collagen and fibronectin: identification of potential adhesins

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Abstract

Paracoccidioidomycosis is caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The extracellular matrix (ECM) plays an important role in regulation of cell adhesion, differentiation, migration and proliferation of cells. An in vitro binding assay of *P. brasiliensis* yeast cells adhering to type I collagen and fibronectin was performed in order to identify novel adhesins. Representational difference analysis (RDA) was employed to identify genes upregulated under adhesion-inducing conditions. Expressed sequence tags (ESTs) from cDNA libraries generated by the RDA technique were analyzed. Genes related to functional categories, such as metabolism, transcription, energy, protein synthesis and fate, cellular transport and biogenesis of cellular components were upregulated. Transcripts encoding the *P. brasiliensis* protein enolase (*Pb*Eno) and the high-affinity cooper transporter (*Pb*Ctr3) were identified and further characterized. The recombinant enolase (r*Pb*Eno) and a synthetic peptide designed for *Pb*Ctr3 were obtained and demonstrated to be able to bind ECM components. Immunofluorescence assays demonstrated that r*Pb*Eno specifically binds to the macrophage surface, reinforcing the role of this molecule in the *P. brasiliensis* interaction with host cells. In addition, upregulation of selected genes was demonstrated by qRT-PCR. In synthesis, the strategy can be useful in characterization of potential *P. brasiliensis* adhesins.

Keywords: Paracoccidioides brasiliensis; Adhesin; RDA; Enolase; Cooper transporter

1. Introduction

Paracoccidioides brasiliensis is the causative agent of paracoccidioidomycosis (PCM), a human systemic mycosis prevalent in South America (Restrepo et al., 2001). In the soil, the fungus grows as a saprobic mycelium, resulting in formation of propagules. After reaching the host, the fungus must convert to the yeast form, a fundamental step in successful establishment of the infection (San-Blas et al., 2002). The mycelial propagules adhere to and invade alveolar cells and the basal lamina, the latter of which is composed of a specialized extracellular matrix (ECM) in which laminin, collagen and fibronectin can be found (Dunsmore and Rannels, 1996; Gonzaléz et al., 2008; Hanna et al., 2000).

Adherence of the pathogens to host cells is considered an essential step in the establishment of infection (Carneiro et al., 2004; Marchais et al., 2005). *P. brasiliensis* has been shown to adhere to ECM proteins. Several studies have established the role of certain *P. brasiliensis* proteins in the adherence process. An antigenic component of *P. brasiliensis*, glycoprotein gp43,

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binds laminin, thereby increasing the pathogenicity of the yeast cells (Vicentini et al., 1994). Proteins with molecular masses of 19 and 32 kDa are present on the fungal surface and interact with laminin, fibronectin and fibrinogen (Gonzaléz et al., 2005). The 32 kDa protein (*Pb*Had32p) was characterized as a hydrolase that influences *P. brasiliensis* pathogenicity (Hernández et al., 2010). In addition, Andreotti et al. (2005) demonstrated that a *P. brasiliensis* 30 kDa protein is able to bind laminin. We characterized several *P. brasiliensis* adhesins such as *Pb*Dfg5p (defective for filamentous growth protein Dfg5p), which was detected by electron microscopy in the cell wall of the fungus and binds laminin, fibronectin and types I and IV collagen (Castro et al., 2008). In addition, triosephosphate isomerase (*Pb*TPI) which binds laminin and fibronectin (Pereira et al., 2007), and glyceraldehyde-3-phosphate dehydrogenase (*Pb*GAPDH), which binds fibronectin, type I collagen and laminin (Barbosa et al., 2006), were found in the *P. brasiliensis* cell wall mediating fungal adherence to in vitro cultured cells. Malate synthase (*Pb*MLS) binds fibronectin and types I and IV collagen and is present in the *P. brasiliensis* cell wall (Neto et al., 2009). In addition, *P. brasiliensis* enolase is a fibronectin and plasminogen binding protein (Donofrio et al., 2009; Nogueira et al., 2010). Therefore, *P. brasiliensis* seems to possess several proteins involved in adhesion, and knowledge of these proteins could advance our understanding of the first steps in establishment of the infection.

To obtain and characterize new molecules involved in the adhesion process in *P. brasiliensis*, we used cDNA representational difference analysis (cDNA-RDA) to identify genes induced during incubation of *P. brasiliensis* yeast cells with ECM components. Fibronectin, a multifunctional extracellular matrix and plasma protein that plays a central role in cell adhesion (Ruoslahti, 1988), and collagens, as the most common matrix molecules (Lyons and Jones, 2007), represent targets for microorganism adherence. Therefore, in this study, we investigated involvement of type I collagen and fibronectin in the adherence process of *P. brasiliensis* and described several putative novel adhesins.

2. Materials and methods

2.1. P. brasiliensis *growth conditions*

P. brasiliensis Pb 01 (ATCC MYA-826) is being studied at our laboratory (Bailão et al., 2006; Barbosa et al., 2006). This isolate was cultivated at 36 °C in Fava-Netto's medium [1% (w/v) peptone; 0.5% (w/v) yeast extract; 0.3% (w/v) proteose peptone; 0.5% (w/v) beef extract; 0.5% (w/v) NaCl; 4% (w/v) glucose; 1% (w/v) agar; pH 7.2] for 4 days.

2.2. Adherence assay on polystyrene flasks

The adherence assays were performed as described by Penalver et al. (1996) with several modifications. Briefly, polystyrene flasks (Corning Ultra-Low Attachment 75 cm^2 rectangular canted-neck cell-culture flask) were coated with type I collagen or fibronectin at 50 μg/ml in coating buffer ($NaHCO_3$, Na_2CO_3, [pH 9.6]) and incubated for 1 h at 37 °C and overnight at 4 °C. The plates were blocked by adding PBS (1 mM $Na_2HPO_4.2H_2O$, 1 mM $NaH_2PO_4.H_2O$, 50 mM NaCl, pH 7.4)−1% (w/v) BSA and washed three times with PBS−0.1% (v/v) Tween 20 before a yeast cell suspension (10^8/ml) in PBS was added. The control yeast cells were incubated in PBS−1% (w/v) BSA. The plates were incubated for 1 h at 37 °C and washed three times with PBS−0.1% (v/v) Tween 20 following RNA isolation.

2.3. RNA isolation

Total RNAs from *P. brasiliensis* were obtained by the Trizol method according to the manufacturer's instructions (GIBCO, Invitrogen, Carlsbard, CA, USA). DNA contamination was extinguished by treating total RNA with RNAse free DNAse (Promega Corporation®). The RNAs were used to construct double-stranded cDNAs.

2.4. Subtractive hybridization and generation of subtracted libraries

Subtractive hybridization was performed as previously described by Bailão et al. (2006). Briefly, 1.0 μg of total RNA was used to produce cDNA. The synthesis of the first strand was performed with SuperScript II reverse transcriptase (Invitrogen Life Technologies); this product was then used as a template to synthesize double-stranded cDNA. The resulting cDNAs were digested with restriction enzyme *Sau3*AI. The subtracted cDNA libraries were constructed using driver cDNAs (from RNAs extracted from the control) and tester cDNAs (synthesized from RNAs extracted from *P. brasiliensis* adhered to type I collagen or fibronectin). The resulting products were purified using a GFX kit (GE Healthcare, Chalfont St. Giles, UK). The tester-digested cDNA was ligated to adapters (a 24-mer annealed to a 12-mer) and amplified by PCR. The amplicons were digested with *Sau3*AI to remove the adapters that had been incorporated into the cDNAs and, after spin-column purification, a new 24-mer adapter was ligated onto the cDNA tester and a different DNA molecule was ligated onto the cDNA driver. The cDNA driver was PCR-amplified and, after cleavage to remove the adapters, it was purified and quantified.

For generation of the differential products, tester and driver cDNAs were mixed, hybridized at 67 °C for 18 h and amplified by PCR with the 24-mer adapter. Two successive rounds of subtraction and PCR amplification using hybridization tester-driver ratios 1:10 and 1:100 were performed. The adapters used for subtractive hybridizations are listed in Table 1 in supplementary material.

After the second subtractive reaction, the final amplified cDNAs were cloned into a pGEM-T Easy vector (Promega, Madison, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. Selected colonies were picked and grown in microliter plates and plasmid DNA was prepared. To generate expressed sequence tags (ESTs), single-pass, 5′-end sequencing of cDNAs by standard fluorescence labeling dye-terminator protocols with T7 flanking vector primer was performed. The samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare) for automated sequencing analysis.

2.5. EST processing pipeline, annotation and sequence analysis

EST sequences were preprocessed using Phred (Ewing and Green, 1998) and Crossmatch programs (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) and were assembled into contigs using CAP3 (Huang and Madan, 1999). All of these tools were integrated in a specific pipeline (http://www.lbm.icb.ufg.br/pipelineUFG/). Only sequences with at least 75 nucleotides and PHRED quality greater than or equal to 20 were considered. ESTs were screened for vector sequences against UniVec data. The clustered sequences were compared using Blast X against the GenBank non-redundant (nr) database from the National Center for Biotechnology Information (NCBI) and the nucleotide database generated from the *P. brasiliensis* structural genome (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). The database sequence matches were considered significant at E-values $\leq 10^{-10}$.

The search for functional categories was performed using the bioinformatic tool Blast2GO that combines, in one application, GO annotation based on similarity searches with statistical analysis and highlight visualization on directed acyclic graphs (Conesa et al., 2005). The Blast2GO annotation algorithm takes multiple parameters into account, such as sequence similarity, BLAST HSP (highest scoring pair) length and e-values, the GO hierarchical structure and GO term evidence codes (Conesa et al., 2005; Götz et al., 2008). The sequences were grouped into functional categories according to the classification of the MIPS functional catalog (Munich Center for Protein Sequences; http://mips.gst.de/).

The in silico prediction of adhesins was performed using the tool Faapred (Fungal adhesin and adhesin-like proteins prediction) hosted at http://bioinfo.icgeb.res.in/faap (Ramana and Gupta, 2010). The protein sequences encoded by RDA products were obtained from the *P. brasiliensis* database (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html) and then loaded onto the software above for predictions.

2.6. Analysis of RNA transcripts by quantitative reverse-transcription PCR (qRT-PCR)

This assay was performed to confirm RDA results and the reliability of our approaches. Total RNAs from *P. brasiliensis* control yeast cells and from yeast cells adhered to type I collagen or fibronectin were obtained as previously described in independent experiments from those used in the RDA. Total RNAs treated with DNAse were reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo $(dT)_{15}$ primer. The qRT-PCR was performed in triplicate with samples from three independent experiments in the StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA). The PCR thermal cycling was 40 cycles of 95 °C for 15 s and 60 °C for 1 min. SYBR Green PCR master mix (Applied Biosystems) was used as the reaction mixture to which were added 10 pmol of each specific primer and 40 ng of template cDNA in a final volume of 20 μl. Melting curve analysis was performed to confirm a single PCR product. The data were normalized with the transcript for α-tubulin amplified in each set of qRT-PCR experiments. A non-template control was included. A cDNA for a relative standard curve was generated by pooling an aliquot from each cDNA sample. The standard curve was serially diluted 1:5 and a standard curve was generated using five samples from the pooled cDNA. Relative expression levels of genes of interest were calculated using the standard curve method for relative quantification (Bookout et al., 2006). The specific primers, both sense and antisense, are described in Table 1 in supplementary material.

2.7. Cloning the cDNA encoding enolase into an expression vector and purification of the recombinant protein

The procedures for obtaining the recombinant protein enolase were performed as previously described (Nogueira et al., 2010). The complete enolase cDNA (GenBank accession number EF558735.1), obtained from a library from yeast cells of *P. brasiliensis* (Costa et al., 2007), was amplified by PCR employing primers, as described in Table 1 of the supplementary material. The PCR product was cloned in-frame with the glutathione S-transferase (GST) coding region of the pGEX-4T3 vector to yield the GST-*Pb*Eno construct. The procedures for obtaining the recombinant protein were performed as previously described (Nogueira et al., 2010).

Bacteria of the *E. coli* strain BL21 pLys, transformed with the GST-*Pb*Eno construct, were grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg/ml) and glucose (20 mM) at 37 °C, 200 rpm. At an A_{600} of 0.6, protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. After centrifugation, *E. coli* bacterial pellets were resuspended in PBS, incubated on ice for 30 min and sonicated on ice 15 times for 60 s each. The GST-*Pb*Eno protein was affinity-purified using glutathione Sepharose 4B (GE Healthcare) according to the manufacturer's protocol, and *Pb*Eno was released from GST-*Pb*Eno by the addition of thrombin (Sigma Aldrich). The cleavage reaction was stopped by freezing the sample at −20 °C. The purity and integrity of the protein were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie Blue staining.

2.8. Affinity ligand assays and dot blot analysis

Far-western assays were carried out as previously described (Barbosa et al., 2006; Castro et al., 2008). The recombinant enolase (r*Pb*Eno) was submitted to SDS-PAGE and blotted onto nitrocellulose membranes. The blotted protein was assayed for laminin, fibronectin, type I and type IV collagen binding, as follows. The blotted membranes were blocked for 4 h with PBS−1% (w/v) BSA and 5% (w/v) milk, incubated with laminin (30 μg/ml), fibronectin (30 μg/ml), type I collagen (20 μg/ml) or type IV collagen (20 μg/ml) diluted in PBS−1% (w/v) BSA for 90 min and washed three times with PBS−0.1% (v/v) Tween 20. The membranes were incubated overnight with the rabbit antibodies anti-laminin, anti-fibronectin, anti-type I collagen or anti-type IV collagen (diluted 1:100). The blots were washed with PBS−0.1% (v/v) Tween 20 and incubated with peroxidase-labeled goat anti-rabbit immunoglobulin (diluted 1:1000) for 2 h. The blots were washed with PBS−0.1% (v/v) Tween 20 and the reactive bands were developed with hydrogen peroxide diaminobenzidine as the chromogenic reagent. As a negative control, r*Pb*Eno was incubated only with peroxidase-labeled goat anti-rabbit immunoglobulin in the absence of the ECM proteins (laminin, fibronectin and type I and IV collagen). An additional control was obtained by incubating r*Pb*Eno with BSA.

A peptide was synthesized based on the deduced sequence of *Pb*Ctr3 (GenBank accession number DQ534496) toward amino acids 90−130 (Dantas et al., 2009), and dot blot analysis was performed to assay the reactivity of this peptide to the ECM proteins. The reactions were performed as described above for the affinity ligand assay.

2.9. Immunofluorescence

J774 A.1 macrophage cells purchased from Banco de Células do Rio de Janeiro (Rio de Janeiro Brazil) were cultured over coverslips in 6-well plates and subjected to an enolase binding assay. Mammalian cells were cultured in RPMI supplemented with interferon gamma (1 U/ml). The medium was removed and the cells were washed 3 times with PBS, fixed for 30 min with cold methanol and air-dried. Either recombinant enolase (350 μg/ml) or 1% BSA (w/v, negative control) in PBS was added and incubated with fixed J774 cells at room temperature for 1 h. After cells were washed 3 times with PBS, mouse enolase antiserum (1:100 dilution) was added. The system was incubated for 1 h at 37 °C and washed 3 times with PBS. The cells were incubated with anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC; 1:100 dilution) for 1 h. The cells were incubated with 50 μM 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining.

2.10. Statistical analysis

The experiments were performed in triplicate with samples in triplicate. The results were presented as means ± standard deviation. The statistical comparisons were performed using Student's *t*-test. Statistical significance was accepted for $P < 0.05$.

3. Results

3.1. Expression profile of P. brasiliensis *yeast cells adhering to type I collagen and fibronectin*

The RDA approach was performed with RNAs obtained under three conditions: (a) *P. brasiliensis* yeast cells adhering to type I collagen; (b) *P. brasiliensis* yeast cells adhering to fibronectin; and (c) control *P. brasiliensis* yeast cells. The first and second conditions were used independently as tester cDNA populations and the third was used as the driver cDNA population. Subtraction hybridization was performed by incubating the driver with each tester. Selection of the cDNAs was achieved by construction of subtracted libraries.

For comparative analysis, the 535 ESTs from the cells adhering to type I collagen were grouped into 65 clusters, represented by 30 contigs and 35 singlets. Most of the annotated ESTs (34%) corresponded to energy production. A high proportion of the ESTs found under type I collagen conditions (55%) exhibited sequence similarity to genes of unknown function or encoding hypothetical proteins (Table 1). A broad view of the nature of adaptations made by *P. brasiliensis* during adherence to type I collagen was obtained by classifying the ESTs into seven groups of functionally related genes (Table 1).

ESTs from cells adhering to fibronectin were grouped in 62 clusters, as represented by 25 contigs and 37 singlets. The analysis of 583 ESTs revealed that most of the annotated ESTs (42%) corresponded to transcripts related to cell rescue, defense and virulence (Table 2), while 31% of the ESTs found under fibronectin-binding conditions did not show similarity to known *P. brasiliensis* genes. The annotated ESTs comprised nine different MIPS categories, indicating a wide range of processes probably involved in *P. brasiliensis* adhesion to fibronectin (Table 2).

The Faapred bioinformatics tool used for identification of fungal adhesins is trained software with different compositional features: namely, amino acid, dipeptide, multiplet fractions, charge and hydrophobic compositions, as well as PSI-Blast (Ramana and Gupta, 2010). In silico adhesin prediction analysis using the Faapred tool returned 16 out of 30 upregulated genes and 27 out from 44 upregulated genes from collagen and fibronectin conditions, respectively (Tables 1 and 2).

3.2. qRT-PCR assays in analysis of gene expression

For further confirmatory data on the expression level from EST redundancy analysis, an assessment of *P. brasiliensis* alcohol dehydrogenase (*Pbadh*), enolase (*Pbeno*), arginine N-methyltransferase (*Pbskb1*), enoyl-CoA hydratase (*Pbenoyl-CoA*), copper transporter (*Pb*ctr3) and heat-shock protein 70 (*Pbhsp70*) was provided by qRT-PCR analysis. *Pbadh*, *Pbeno* and *Pbskb1* were induced in yeast cells adhering to type I collagen and fibronectin (Fig. 1A), as demonstrated in the RDA. *Pbenoyl-CoA* was induced in yeast

cells adhering to collagen (Fig. 1B), and *Pb*ctr3 and *Pbhsp70* were induced in yeast cells adhering to fibronectin (Fig. 1C), confirming RDA.

3.3. *r*Pb*Eno and* Pb*Ctr3 bind to matrix proteins*

We selected the transcripts encoding enolase (*Pb*eno) and the high-affinity cooper transporter (*Pb*ctr3) for testing the ability of the cognate proteins to adhere to ECM components in vitro. The criterion for selection was the predicted cell wall localization of both proteins, as previously described (Dantas et al., 2009; Nogueira et al., 2010). For that, the full-length cDNA encoding enolase consisted of 1684 bp with an open reading frame encoding 438 amino acids with a calculated molecular mass of 47 kDa. cDNA encoding *P. brasiliensis* enolase was cloned into the expression vector pGEX-4T-3 to obtain the recombinant fusion protein in *E. coli*. After induction with IPTG, a recombinant protein was detected in bacterial lysates (Fig. 2A, lane 2). The fusion protein was affinity-purified and r*Pb*Eno was obtained by digestion with thrombin (Fig. 2A, lane 3).

The ability of r*Pb*Eno to bind laminin, fibronectin and type I and IV collagens was determined by far-western blotting assays, as shown in Fig. 2B. r*Pb*Eno exhibited the ability to bind to laminin (lane 3), fibronectin (lane 4) and type I collagen (lane 5). There was no detectable reaction with type IV collagen (lane 6). Negative controls were obtained by incubating r*Pb*Eno in the absence of the ECM proteins (lane 1), and by using BSA (lane 2).

In addition, the synthetic peptide (*Pb*Ctr3) (Fig. 2C), reacted with type I collagen (lane 2), type IV collagen (lane 3) and fibronectin (lane 4). There was no reactivity with BSA (negative control) (lane 1) or laminin (lane 5).

Table 1
Annotated ESTs with high abundance in yeast cells during adhesion to collagen versus control yeast cells.

Functional category	Gene product	Best hit/GenBank accession number* or *P. brasiliensis* genome locus**	e-Value	Number of occurrences
Metabolism	Acetamidase[b]	*P. brasiliensis*/PAAG_03626.1**	1e−55	12
	Transketolase	*P. brasiliensis*/PAAG_04444.1**	1e−55	5
	Enoyl-CoA hydratase	*P. brasiliensis*/PABG_02862.1**	1e−38	2
	Mitochondrial protein potentially involved in regulation of respiratory metabolism	*Saccharomyces cerevisiae*/NP_690845.1*	3e−11	8
	Alcohol dehydrogenase[a]	*P. brasiliensis*/PAAG_04541.1**	1e−51	1
Energy	NADH dehydrogenase[b]	*P. brasiliensis*/PAAG_04760.1**	1e−26	176
	Enolase[a,b]	*P. brasiliensis*/PAAG_00771.1**	1e−56	3
Transcription	Transcription factor MetR	*P. brasiliensis*/PAAG_04371.1**	1e−14	5
	Endoribonuclease ysh1 (Bzip)	*P. brasiliensis*/PAAG_08788.1**	1e−76	6
	SWI/SNF transcription activation complex subunit	*P. brasiliensis*/PAAG_06542.1**	1e−52	1
	Protein Kruppel[b]	*P. brasiliensis*/PAAG_06709.1**	1e−27	1
	Pre mRNA splicing factor prp1	*P. brasiliensis*/PAAG_00995.1**	1e−26	1
Protein binding	FAD-linked sulfhydryl oxidase	*P. brasiliensis*/PAAG_06132.1**	1e−35	2
	Cytosolic Fe-S cluster assembling factor NBP35[b]	*P. brasiliensis*/PAAG_03944.1**	1e−112	1
Cell cycle and DNA processing	DNA polymerase epsilon subunit c[b]	*P. brasiliensis*/PAAG_00002.1**	1e−10	4
Cell rescue virulence	Hsp98/Hsp104	*P. brasiliensis*/PAAG_02130.1**	1e−49	2
Protein fate	Arginine N-methyltransferase skb1[a,b]	*P. brasiliensis*/PAAG_02402.1**	1e−85	3
Unclassified proteins	Conserved hypothetical protein[b]	*P. brasiliensis*/PADG_08537.1**	5e−41	32
	Conserved hypothetical protein[b]	*P. brasiliensis*/PAAG_08039.1**	1e−19	2
	Conserved hypothetical protein[b]	*P. brasiliensis*/PABG_01516.1**	1e−13	1
	Conserved hypothetical protein	*P. brasiliensis*/PAAG_04760.1**	1e−26	1
	Conserved hypothetical protein	*P. brasiliensis*/PAAG_01303.1**	1e−34	1
	Conserved hypothetical protein	*P. brasiliensis*/PAAG_07033.1**	1e−13	16
	Conserved hypothetical protein[b]	*P. brasiliensis*/PABG_03557.1**	1e−34	2
	Conserved hypothetical protein[b]	*P. brasiliensis*/PABG_07127.1**	1e−18	6
	Hypothetical protein[b]	*P. brasiliensis*/PABG_06807.1**	1e−27	167
	Hypothetical protein[b]	*P. brasiliensis*/PAAG_07288.1**	1e−36	49
	Hypothetical protein	*P. brasiliensis*/PABG_01874.1**	1e−64	4
	Hypothetical protein[b]	*P. brasiliensis*/PAAG_03580.1**	1e−49	3
	Hypothetical protein[b]	*P. brasiliensis*/PAAG_02061.1**	1e−19	2
No significant similarity found				2

[a] Transcripts overexpressed in the presence of type I collagen and fibronectin.
[b] Putative adhesins predicted by Faadpred in silico analysis.

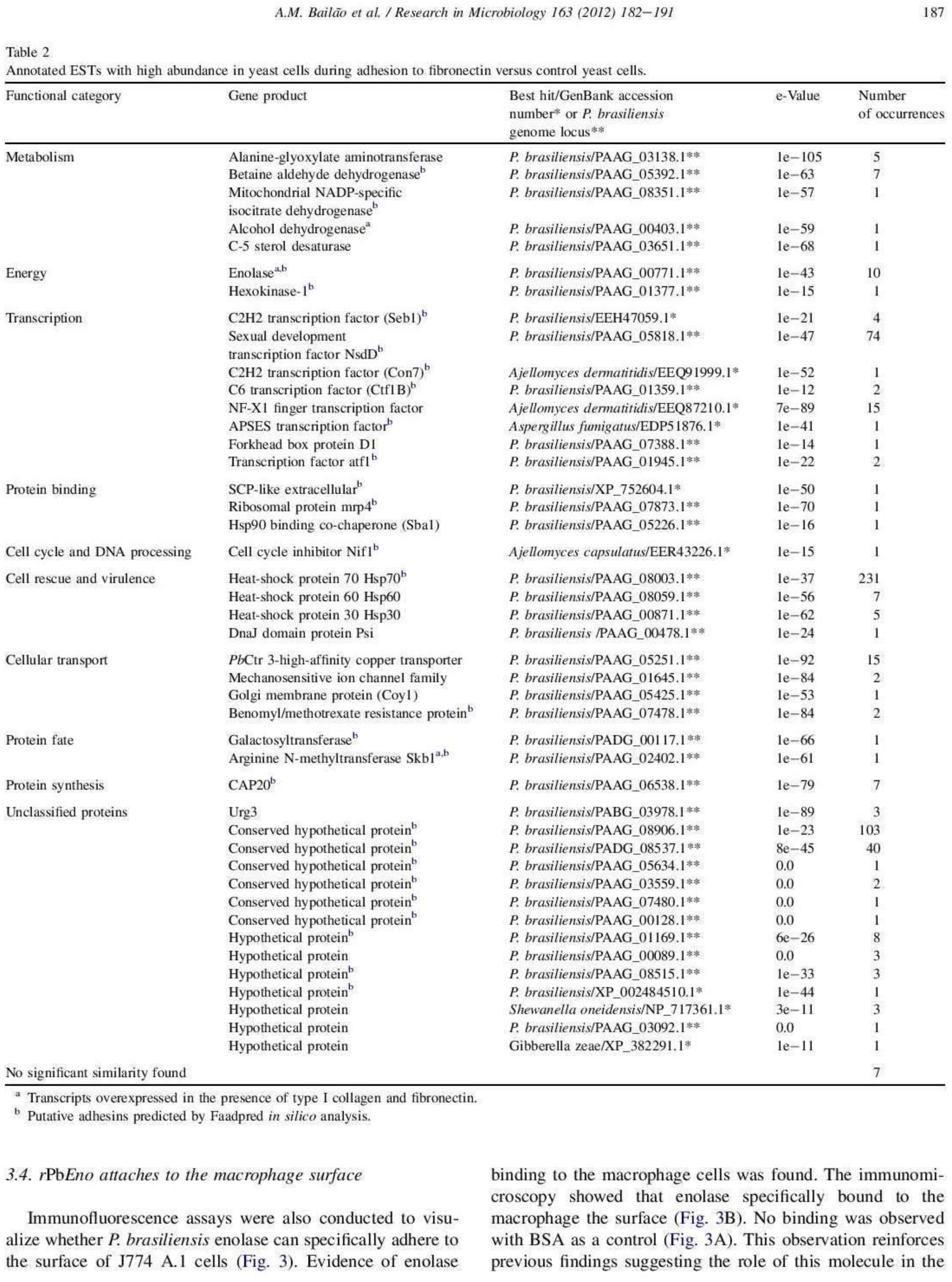

Table 2
Annotated ESTs with high abundance in yeast cells during adhesion to fibronectin versus control yeast cells.

Functional category	Gene product	Best hit/GenBank accession number* or *P. brasiliensis* genome locus**	e-Value	Number of occurrences
Metabolism	Alanine-glyoxylate aminotransferase	*P. brasiliensis*/PAAG_03138.1**	1e−105	5
	Betaine aldehyde dehydrogenase[b]	*P. brasiliensis*/PAAG_05392.1**	1e−63	7
	Mitochondrial NADP-specific isocitrate dehydrogenase[b]	*P. brasiliensis*/PAAG_08351.1**	1e−57	1
	Alcohol dehydrogenase[a]	*P. brasiliensis*/PAAG_00403.1**	1e−59	1
	C-5 sterol desaturase	*P. brasiliensis*/PAAG_03651.1**	1e−68	1
Energy	Enolase[a,b]	*P. brasiliensis*/PAAG_00771.1**	1e−43	10
	Hexokinase-1[b]	*P. brasiliensis*/PAAG_01377.1**	1e−15	1
Transcription	C2H2 transcription factor (Seb1)[b]	*P. brasiliensis*/EEH47059.1*	1e−21	4
	Sexual development transcription factor NsdD[b]	*P. brasiliensis*/PAAG_05818.1**	1e−47	74
	C2H2 transcription factor (Con7)[b]	*Ajellomyces dermatitidis*/EEQ91999.1*	1e−52	1
	C6 transcription factor (Ctf1B)[b]	*P. brasiliensis*/PAAG_01359.1**	1e−12	2
	NF-X1 finger transcription factor	*Ajellomyces dermatitidis*/EEQ87210.1*	7e−89	15
	APSES transcription factor[b]	*Aspergillus fumigatus*/EDP51876.1*	1e−41	1
	Forkhead box protein D1	*P. brasiliensis*/PAAG_07388.1**	1e−14	1
	Transcription factor atf1[b]	*P. brasiliensis*/PAAG_01945.1**	1e−22	2
Protein binding	SCP-like extracellular[b]	*P. brasiliensis*/XP_752604.1*	1e−50	1
	Ribosomal protein mrp4[b]	*P. brasiliensis*/PAAG_07873.1**	1e−70	1
	Hsp90 binding co-chaperone (Sba1)	*P. brasiliensis*/PAAG_05226.1**	1e−16	1
Cell cycle and DNA processing	Cell cycle inhibitor Nif1[b]	*Ajellomyces capsulatus*/EER43226.1*	1e−15	1
Cell rescue and virulence	Heat-shock protein 70 Hsp70[b]	*P. brasiliensis*/PAAG_08003.1**	1e−37	231
	Heat-shock protein 60 Hsp60	*P. brasiliensis*/PAAG_08059.1**	1e−56	7
	Heat-shock protein 30 Hsp30	*P. brasiliensis*/PAAG_00871.1**	1e−62	5
	DnaJ domain protein Psi	*P. brasiliensis* /PAAG_00478.1**	1e−24	1
Cellular transport	*Pb*Ctr 3-high-affinity copper transporter	*P. brasiliensis*/PAAG_05251.1**	1e−92	15
	Mechanosensitive ion channel family	*P. brasiliensis*/PAAG_01645.1**	1e−84	2
	Golgi membrane protein (Coy1)	*P. brasiliensis*/PAAG_05425.1**	1e−53	1
	Benomyl/methotrexate resistance protein[b]	*P. brasiliensis*/PAAG_07478.1**	1e−84	2
Protein fate	Galactosyltransferase[b]	*P. brasiliensis*/PADG_00117.1**	1e−66	1
	Arginine N-methyltransferase Skb1[a,b]	*P. brasiliensis*/PAAG_02402.1**	1e−61	1
Protein synthesis	CAP20[b]	*P. brasiliensis*/PAAG_06538.1**	1e−79	7
Unclassified proteins	Urg3	*P. brasiliensis*/PABG_03978.1**	1e−89	3
	Conserved hypothetical protein[b]	*P. brasiliensis*/PAAG_08906.1**	1e−23	103
	Conserved hypothetical protein[b]	*P. brasiliensis*/PADG_08537.1**	8e−45	40
	Conserved hypothetical protein[b]	*P. brasiliensis*/PAAG_05634.1**	0.0	1
	Conserved hypothetical protein[b]	*P. brasiliensis*/PAAG_03559.1**	0.0	2
	Conserved hypothetical protein[b]	*P. brasiliensis*/PAAG_07480.1**	0.0	1
	Conserved hypothetical protein[b]	*P. brasiliensis*/PAAG_00128.1**	0.0	1
	Hypothetical protein[b]	*P. brasiliensis*/PAAG_01169.1**	6e−26	8
	Hypothetical protein	*P. brasiliensis*/PAAG_00089.1**	0.0	3
	Hypothetical protein[b]	*P. brasiliensis*/PAAG_08515.1**	1e−33	3
	Hypothetical protein[b]	*P. brasiliensis*/XP_002484510.1*	1e−44	1
	Hypothetical protein	*Shewanella oneidensis*/NP_717361.1*	3e−11	3
	Hypothetical protein	*P. brasiliensis*/PAAG_03092.1**	0.0	1
	Hypothetical protein	Gibberella zeae/XP_382291.1*	1e−11	1
No significant similarity found				7

[a] Transcripts overexpressed in the presence of type I collagen and fibronectin.
[b] Putative adhesins predicted by Faadpred *in silico* analysis.

*3.4. r*Pb*Eno attaches to the macrophage surface*

Immunofluorescence assays were also conducted to visualize whether *P. brasiliensis* enolase can specifically adhere to the surface of J774 A.1 cells (Fig. 3). Evidence of enolase binding to the macrophage cells was found. The immunomicroscopy showed that enolase specifically bound to the macrophage the surface (Fig. 3B). No binding was observed with BSA as a control (Fig. 3A). This observation reinforces previous findings suggesting the role of this molecule in the

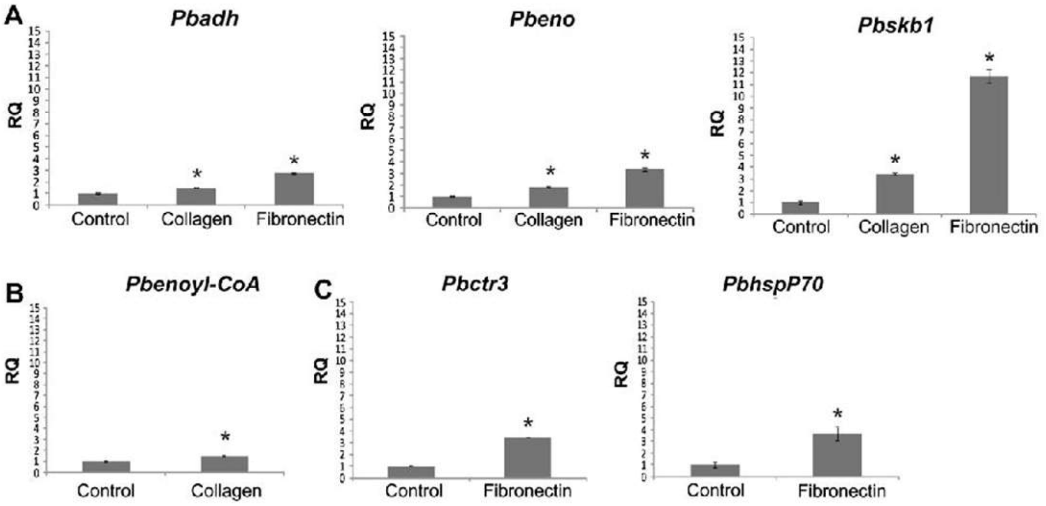

Fig. 1. Average gene expression of *Pbadh*, *Pbeno*, *Pbskb*1, *Pbenoyl-CoA*, *Pbctr3* and *Pbhsp70*, as determined by quantitative real-time RT-PCR. (A) qRT-PCR plot of *Pbadh*, *Pbeno* and *Pbskb*1 expression levels in yeast cells adhering to type I collagen and fibronectin. (B) qRT-PCR plot of *Pbenoyl-CoA* expression levels in yeast cells adhering to type I collagen. (C) qRT-PCR plot of *Pbctr3* and *Pbhsp70* expression levels in yeast cells adhering to fibronectin. The values of expression were standardized using values of expression of the constitutive gene encoding α-tubulin. The expression level was calculated by the relative standard curve method. The standard deviations are presented from three independent experiments. *, Significantly different from the control, at a *P*-value of <0.05.

attaching process between *P. brasiliensis* and host cells (Nogueira et al., 2010).

4. Discussion

Our objective in the present work was to uncover potential adhesins that could be expressed during the adhesion process of *P. brasiliensis*. For this purpose, in vitro adherence assays to extracellular matrix proteins were performed. The RDA assays allowed identification of 69 upregulated genes during fibronectin and collagen adhering conditions. Three genes were found in both conditions. Among the identified transcripts, several were identified as coding for previously characterized adhesins such as alcohol dehydrogenase (ADH), Hsp60 and Hsp70. Many differentially expressed transcripts detected in this work, such as C-5 sterol desaturase, cap20 protein, high-affinity copper transporter, hexokinase and transketolase, had already been described as upregulated genes in yeast cells derived from models of infection (Bailão et al., 2006; Costa et al., 2007). The induced expression of putative virulence factors indicates that the presence of ECM components may be a stimulus to trigger mechanisms to adapt to the host milieu. Among the RDA differential products, many transcripts encoding hypothetical proteins were isolated. Bioinformatics-based analysis confirmed that most RDA products are predicted to be adhesin-like molecules and many of them are proteins with unknown function.

By screening a cDNA expression library of *Candida albicans* yeast cells with polyclonal antiserum to human fibronectin, Klotz et al. (2001) isolated cDNA clones that encode ADH, suggesting that this protein is found on the cell surface of this fungus and could be a receptor for fibronectin. Also, Crowe et al. (2003), in an attempt to identify *C. albicans* proteins involved in plasminogen binding, identified ADH in cell wall protein extracts of this fungus. Upregulation of transcripts encoding *P. brasiliensis* ADH during contact with ECM components suggests the role of this protein in adhesion of yeast cells to host tissues.

The transcript-encoding enolase was induced under both studied conditions. Several studies had demonstrated the role of enolase as an ECM binding protein. This molecule is a cell surface protein in *Staphylococcus aureus* and mediates binding of this microorganism to laminin, potentially playing a critical role in its pathogenesis (Carneiro et al., 2004). In addition, previous works showed the surface localization of *Streptococcus suis* enolase and its ability to bind to fibronectin (Esgleas et al., 2008) and to Hep-2 cell surface (Feng et al., 2009). Using a proteomic approach, Chen et al. (2011) showed that *S. suis* enolase is a protein that binds to macrophage surface molecules. Moreover, Castaldo et al. (2009), using immune electron microscopy also demonstrated the cell surface localization of *Lactobacillus plantarum* enolase, where it can bind fibronectin and mediate adhesion of this commensal bacterium to human intestinal cells. Regarding *P. brasiliensis*, *Pb*Eno was previously described as a fibronectin-binding protein that mediates the interaction between the fungus and pulmonary epithelial cells, A549 (Donofrio et al., 2009). Our recent studies demonstrated the potential contribution of *Pb*Eno as a virulence factor for *P. brasiliensis*. In fact, in *P. brasiliensis*, surface-associated enolase was documented and shown to bind host plasminogen. Moreover, plasminogen-coated *P. brasiliensis* yeast cells are capable of degrading purified fibronectin, providing in vitro evidence for the generation of plasmin on the fungus surface. In addition,

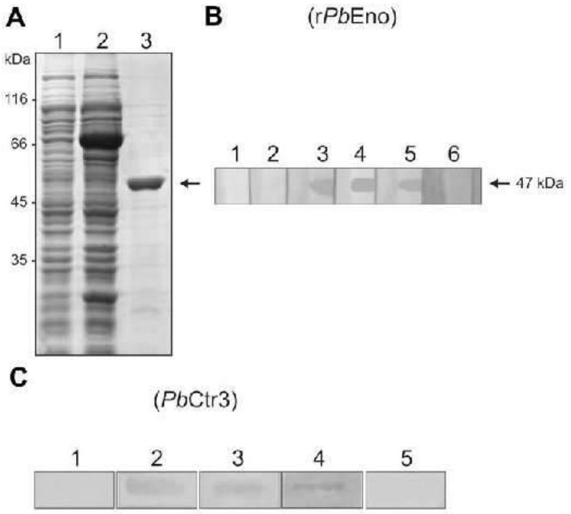

Fig. 2. Binding of *Pb*Eno and *Pb*Ctr3 to extracellular matrix components. (A) SDS-PAGE analysis of *P. brasiliensis* recombinant enolase (r*Pb*Eno). *E. coli* cells harboring the pGEX-4T-3-enolase plasmid were grown at 37 °C to an A_{600} of 0.6 and harvested before (lane 1) and after (lane 2) 16 h incubation at 15 °C with 0.1 mM IPTG. The cells were lysed by extensive sonication. Lane 3, purified r*Pb*Eno (after cleavage with thrombin). The protein extracts were fractionated by one-dimensional gel electrophoresis and stained by Coomassie Blue. (B) Recombinant enolase (0.5 μg) was subjected to SDS-PAGE and electroblotted. The membranes were reacted with laminin (lane 3), fibronectin (lane 4), type I collagen (lane 5) and type IV collagen (lane 6) and were subsequently incubated with rabbit IgG anti-laminin, anti-fibronectin, anti-type I collagen and anti-type IV collagen antibodies, respectively. The use of peroxidase-conjugated anti-rabbit IgG revealed the reactions. The negative controls were obtained by incubating the r*Pb*Eno with no ECM component (lane 1) and using BSA (lane 2). (C) Reactivity of the synthetic peptide from *Pb*Ctr3 with type I collagen (lane 2), type IV collagen (lane 3), fibronectin (lane 4) or laminin (lane 5). The negative control was obtained by using BSA (lane 1).

recombinant enolase promoted an increase in the association of *P. brasiliensis* with host cells in ex vivo models of infection (Nogueira et al., 2010). The ability of *Pb*Eno to bind to ECM components and to the macrophage surface, as demonstrated by far-western and immunofluorescence in the present work, may account for the molecule's effect in promoting *P. brasiliensis* adhesion to host cells. Although these results point to a contribution by *Pb*Eno to *P. brasiliensis* pathogenesis, studies have been hampered by the lack of a standardized protocol for generation of *P. brasiliensis* knockout mutants and by the fact that the enolase gene is essential.

Molecular chaperones were upregulated during *P. brasiliensis* in vitro adhesion to fibronectin. Chaperones had been detected at the surface of microorganisms, supporting their potential role in adhesion. Specifically, Batista et al. (2006) reported the presence of a member of the J-domain protein family, Mdj1, at the cell surface of *P. brasiliensis*. Hsp60, which has been detected in small clusters at discrete points on the *Histoplasma capsulatum* cell wall, has been shown to mediate attachment of the fungus to macrophages via CD11/CD18 receptors (Long et al., 2003). *Helicobacter pylori* Hsp70 was found at the cell surface and mediates adhesion of the bacteria to glycolipids found in the stomach tissue (Huesca et al., 1998). Likewise, Hsp30, Hsp60 and Hsp70 were found in secretory vesicles in *H. capsulatum* (Albuquerque et al., 2008), suggesting a secretory route for such molecules.

The high-affinity copper transporter is a key molecule related to homeostasis of copper to fungal pathogens. The capacity for copper uptake by pathogenic microorganisms is considered to be a virulence factor because the availability of this metal is low in host tissues (Silva et al., 2011). The impairment of copper uptake in *Cryptococcus neoformans* led to diminished fungal burden in a mouse model of infection (Waterman et al., 2007). The *Pb*ctr3 transcript was induced in *P. brasiliensis* yeast cells derived from infected tissues (Bailão et al., 2006) and was also recognized by sera from PCM patients (Dantas et al., 2009), suggesting its role in the infectious process. The transcript encoding *Pb*Ctr3 was induced in yeast cells in contact with fibronectin. In addition, *Pb*Ctr3 synthetic peptide was able to bind to fibronectin, type I collagen and type II collagen. Taken together, these results suggest that adhesion could be a secondary function of Ctr3, although further investigations are necessary to elucidate this new function. Although this molecule has not been described before as an ECM binding

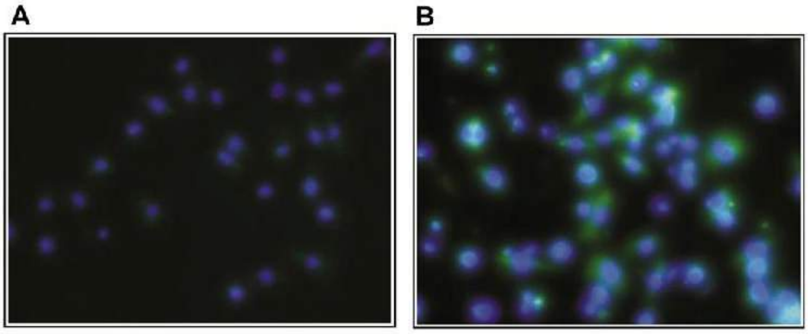

Fig. 3. Binding of r*Pb*Eno to the macrophage surface. Immunofluorescence analysis showing binding of the recombinant enolase to J774 A.1 mouse macrophage cells. (B) Blue indicates the macrophage nucleus and green indicates enolase bound to the surface of the macrophage. (A) The negative control was performed with an unrelated protein (BSA). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

component, its probable localization at the cell surface should enable its binding capacity.

Extracellular matrix in the host tissues provides structural support, compartmentalizes tissues, serves as a physical barrier and is permeable to many compounds. Fibronectin and collagen are major components of the protein content of ECM (Dunsmore and Rannels, 1996). These proteins are the main targets enabling pathogenic microorganisms to attach and invade host tissues by means of adhesins. RDA products obtained from *P. brasiliensis* incubated with fibronectin and collagen revealed many specifically upregulated transcripts. Those findings likely indicate that different ECM components elicit specific pathways that permit a *P. brasiliensis* adaptation mechanism in host tissues. Studies had demonstrated that *C. albicans* is able to respond to very subtle differences in the environment during adhesion to various growth substrates (Sohn et al., 2006; Zakikhany et al., 2007).

In conclusion, this study enabled identification of proteins that may be involved in the adhesion process of *P. brasiliensis*. Indeed, a number of these proteins have already been described in the pathogenesis of this and other microorganisms and elucidation of the role of hypothetical proteins could reveal more information regarding molecules involved in adherence and pathogenesis.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.resmic.2012.01.004.

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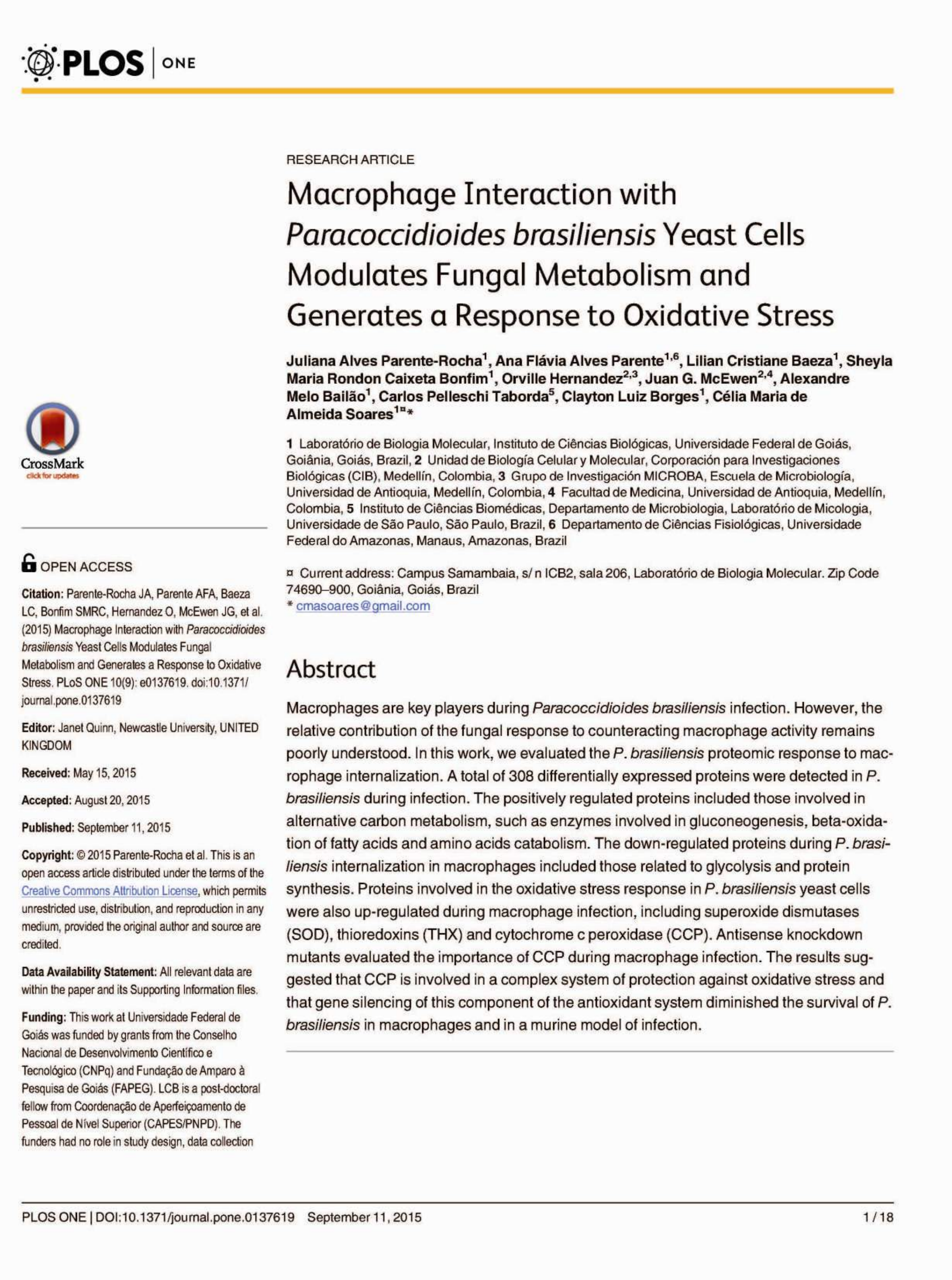
Anexo 2 –



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RESEARCH ARTICLE

Macrophage Interaction with *Paracoccidioides brasiliensis* Yeast Cells Modulates Fungal Metabolism and Generates a Response to Oxidative Stress

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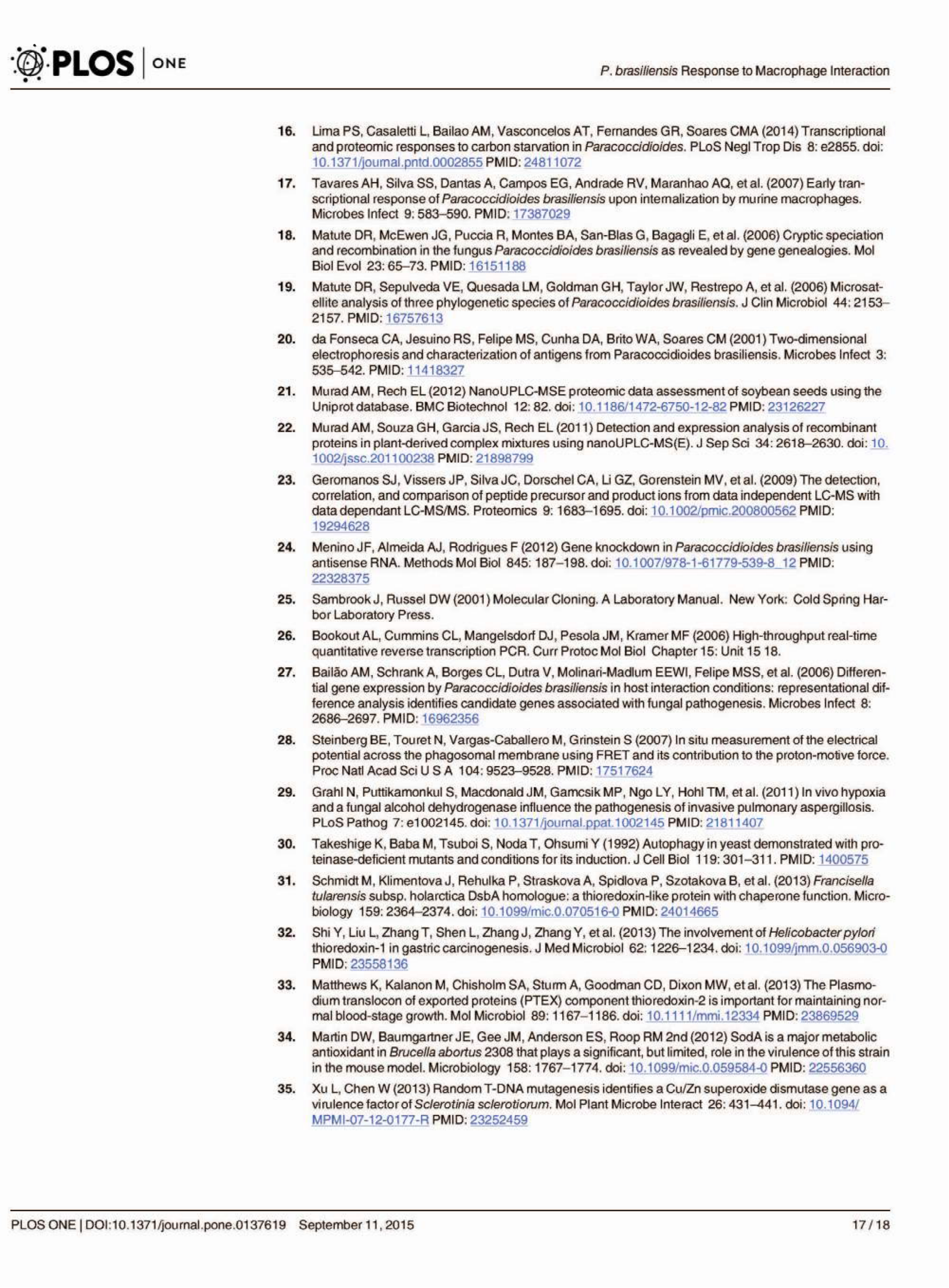
Abstract

Macrophages are key players during *Paracoccidioides brasiliensis* infection. However, the relative contribution of the fungal response to counteracting macrophage activity remains poorly understood. In this work, we evaluated the *P. brasiliensis* proteomic response to macrophage internalization. A total of 308 differentially expressed proteins were detected in *P. brasiliensis* during infection. The positively regulated proteins included those involved in alternative carbon metabolism, such as enzymes involved in gluconeogenesis, beta-oxidation of fatty acids and amino acids catabolism. The down-regulated proteins during *P. brasiliensis* internalization in macrophages included those related to glycolysis and protein synthesis. Proteins involved in the oxidative stress response in *P. brasiliensis* yeast cells were also up-regulated during macrophage infection, including superoxide dismutases (SOD), thioredoxins (THX) and cytochrome c peroxidase (CCP). Antisense knockdown mutants evaluated the importance of CCP during macrophage infection. The results suggested that CCP is involved in a complex system of protection against oxidative stress and that gene silencing of this component of the antioxidant system diminished the survival of *P. brasiliensis* in macrophages and in a murine model of infection.



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and cytochrome c peroxidase (ccp). Transcript levels were measured using quantitative RT-PCR. Data were normalized to the beta tubulin protein transcript and are presented as fold change calculated based on the rate of macrophage interaction to control condition. The Student's t-test was used for statistical comparisons. Error bars represent the standard deviation from three biological replicates, while * represents $p \leq 0.05$.
(PDF)

Author Contributions

Conceived and designed the experiments: CMAS JAPR. Performed the experiments: JAPR AFAP LCB SMRCB OH AMB CLB. Analyzed the data: CMAS JAPR AFAP LCB OH AMB CLB JGM CPT. Contributed reagents/materials/analysis tools: CMAS CPT. Wrote the paper: CMAS JAPR AFAP OH AMB CLB JGM.

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Autophagy is presumably induced in *P. brasiliensis* upon macrophage infection. Autophagy is a vacuolar trafficking pathway that targets subcellular constituents to the vacuole for degradation and recycling; carboxypeptidase Y, vacuolar aminopeptidases and aspartyl protease are involved in this process [38]. The up-regulation of proteins, described as virulence factor in other pathogens, during *P. brasiliensis* interaction with macrophages suggests that these proteins may be important during infection of the human host by *P. brasiliensis*.

Taken together our results show that *P. brasiliensis* responds to several stress conditions once inside macrophages, including glucose deprivation and oxidative stress. The fungal response to glucose deprivation includes a metabolic shift from glycolysis to gluconeogenesis in which glucose precursors are provided by the catabolism of amino acids, as demonstrated by our proteomic analysis. We also assessed the contribution of the fungal oxidative stress response mediated by cytochrome c peroxidase for the survival of *P. brasiliensis* using knockdown strains. According to our data, the enzyme plays a relevant role in fungal survival inside macrophages, and therefore can be described as a virulence factor.

Supporting Information

S1 File. Up-regulated proteins of *P. brasiliensis* during macrophage infection in J774 1.6 cells.
(DOCX)

S2 File. Down-regulated proteins of *P. brasiliensis* during macrophage infection in J774 1.6 cells.
(DOCX)

S3 File. NanoUPLC-MSE data quality analysis. PepFrag1 and PepFrag2 correspond to the peptides matches compared to the database by PLGS, VarMod corresponds to variable modifications, In Source corresponds to fragmentation that occurred in the ionization source, Missed Cleavage indicates the missed cleavage performed by trypsin and Neutral loss HO and NH correspond to water and ammonia precursor losses.
(PDF)

S4 File. Mass error of the identified fragments. The number of identified fragments according to the error range (x-axis).
(PDF)

S5 File. Detection dynamic range. Quantified fragments were sorted according to the fragment amount (Fmol) and plotted in the graphics as grey circles. Standard protein was indicated by red circle. A protein with a low coefficient of variance between samples was used to normalize the expression data and allow comparisons of the control and *P. brasiliensis* data from infected macrophage.
(PDF)

S6 File. Functional categorization of *P. brasiliensis*-regulated proteins during macrophage infection. (A) Abundance (%) of upregulated *P. brasiliensis* proteins during the interaction with macrophages in agreement with their biological functions. (B) Abundance (%) of down-regulated *P. brasiliensis* proteins during the interaction with macrophages in agreement with their biological functions.
(PDF)

S7 File. Quantification of transcripts encoding proteins that were up-regulated during macrophage infection. Transcript levels of genes encoding fructose 1,6 biphosphatase (pbase)

To investigate the effect of the CCP gene on *P. brasiliensis* during infection, we infected BALB/c mice with the *Pb*ccp-aRNA1 strain and compared the results to mice infected with the WT and EV strains. A strong decrease in fungal survival was observed in the livers and spleens of mice infected with the silenced strain (Fig 5). In contrast, no significant differences in fungal burdens in the spleens and livers were detected in mice infected with the WT and EV strains. The results suggest that CCP is important for the establishment of infection by *P. brasiliensis*.

Discussion

In this work, for the first time, we performed a proteomic analysis of the response of *P. brasiliensis* upon interaction with macrophages. A total of 308 proteins were identified as up- or down-regulated in *P. brasiliensis* upon macrophage interaction. This number corresponds to proteins detected in at least two of three biological replicates, and presenting at least ± 50% differences in abundance.

The metabolic changes detected in *P. brasiliensis* reflect how the yeast cells sense the hostile environment in macrophages. Upon phagocytosis by macrophages, *P. brasiliensis* activates responses related to the synthesis of glucose by gluconeogenesis, amino acid catabolism rendering precursors of glucose, and the utilization of fatty acids by beta-oxidation. Additionally, we observed the induction of proteins and enzymes related to ROS detoxification. The results indicated that the anaplerotic precursors for glucose are most likely provided by the carbon backbones released from the amino acid degradation pathways. Enzymes related to glutamate (glutamate dehydrogenase), alanine (alanine glyoxylate aminotransferase) and aspartate (aspartate aminotransferase) catabolism were induced, indicating a possible increase in these catabolic pathways that may result in an enrichment of glucose precursors such as pyruvate, fumarate and oxaloacetate. Ethanol production could be increased, based on the induction of pyruvate decarboxylase and alcohol dehydrogenase and could lead to fungal survival inside the macrophage because ethanol production contributes to pathogenesis [29]. In this way, *P. brasiliensis* might remodel its metabolism to recycle its own carbon-containing molecules. The data suggest that gluconeogenesis play an important role in the adaptive responses to phagocytosis. The induction of gluconeogenesis and amino acid degradation enzymes suggest that *P. brasiliensis* can use carbon backbones of amino acids to synthesize glucose, presumably from the host.

Phagocytic cells generate ROS to eliminate fungi, which are very efficient pathogens in responding to oxidative stress [12]. A number of proteins and transcripts were involved in antioxidant defense systems have been described in *P. brasiliensis in vitro* and upon macrophage phagocytosis [14,17]. The fungus induces the accumulation of detoxifying enzymes such as superoxide dismutases, cytochrome c peroxidase and thioredoxins in response to H_2O_2 [14]. Of special note, in this work we demonstrated that the cytochrome c peroxidase (CCP) plays a role in the *P. brasiliensis* response to oxidative stress during interaction with macrophage cells and infection in a murine model. We previously demonstrated that CCP promotes *Paracoccidioides* sp. protection against nitrosative stress, *in vitro*, as demonstrated by the sensitivity of the *Pb*ccp-aRNA1 strain to S-nitrosoglutathione, (GSNO), indicating an interface of the role played by CCP in oxidative and nitrosative stress responses [15]. The CCP protein is related to the oxidative stress response in other fungi, such as *Cryptococcus neoformans*. A CCP mutant in *C. neoformans* presented a reduction in intracellular growth when cultured with macrophages [36]. Based on our results, cytochrome c peroxidase can be considered a virulence factor because protein silencing promoted a decrease in the number of recovered fungi in macrophages and in an animal model.

Other potential virulence factors induced during macrophage infection were detected in our analysis in *P. brasiliensis* following macrophage infection.

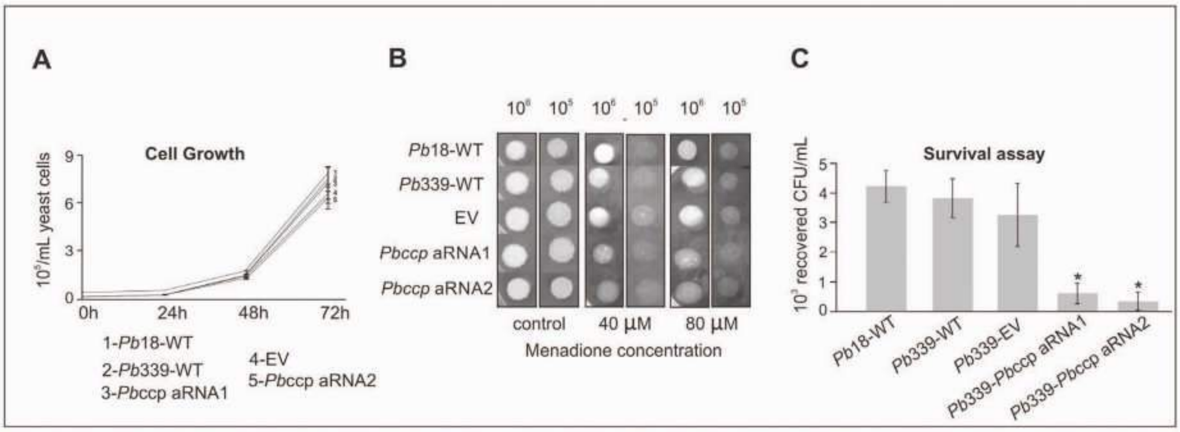

Fig 4. Evaluation of the cell growth, sensitivity of the *Pbccp*-aRNA mutant to oxidative stress and survival in macrophages. (A) Curve of cell growth in BHI medium with the strains *Pb*18-WT (wild type *Pb*18 strain), *Pb*339-WT (wild type *Pb*339 strain), EV: yeast cells (*Pb*339 strain containing the empty vector); *Pbccp*-aRNA1 and *Pbccp*-aRNA2: independent colonies of yeast cells (*Pb*339 strain) containing the cassette with the CCP-AS fragment. **(B)**The *Pbccp*-aRNA sensitivity to oxidative stress was examined in the presence of 40 µM and 80 µM of menadione. *P. brasiliensis Pb*18-WT, *Pb*339-WT and EV were used as controls. **(C)** Interaction assay of *P. brasiliensis* and macrophage cells. The experiments were performed in biological triplicates. *Pb*18-WT: wild type (*Pb*18 strain); *Pb*339-WT: wild type (*Pb*339) EV: yeast cells (*Pb*339 strain) containing the empty vector without CCP-AS; *Pbccp*-aRNA1 and *Pbccp*-aRNA2: independent colonies of yeast cells (*Pb*339 strain) containing the cassette with the CCP-AS fragment. The asterisk denotes $p < 0.01$ (Student's t-test).

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after 24 hours of macrophage infection is depicted in Fig 4, Panel C. The control strains include two isolates of *P. brasiliensis* (*Pb*18-WT and *Pb*339-WT) and EV, and showed no significant differences in the number of CFUs recovered from macrophages. In contrast, the number of CFUs recovered from the CCP knockdown strains (*Pbccp*-aRNA1 and *Pbccp*-aRNA2) was severely reduced, suggesting the importance of the CCP protein during *P. brasiliensis* phagocytosis by macrophage cells.

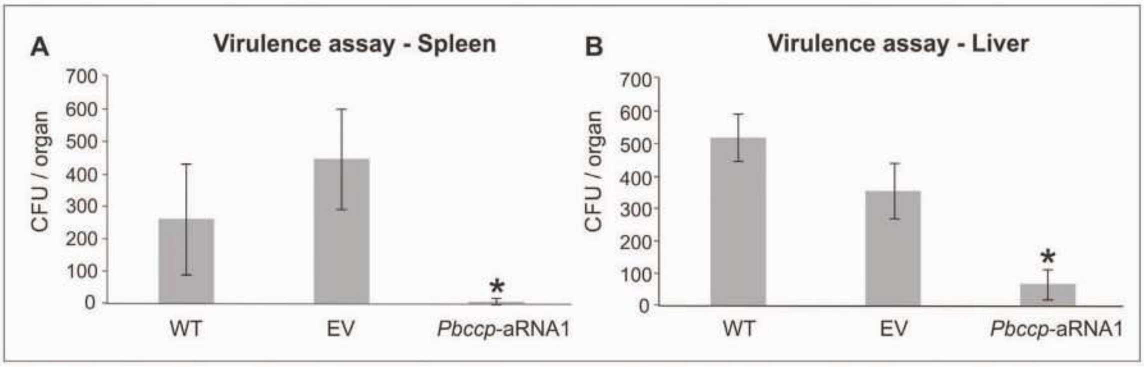

Fig 5. Virulence of the *Pbccp*-aRNA mutant in the liver and spleen of infected BALB/c mice. Colony forming units recovered from the spleen (A) and liver (B) of mice infected with *P. brasiliensis* wild type (WT), *P. brasiliensis* containing the empty vector (EV) and the silenced mutant *Pbccp*-aRNA1. The experiments were performed in biological triplicates. Error bars represent the standard deviation from biological replicates, while * represents *p*-values < 0.01.

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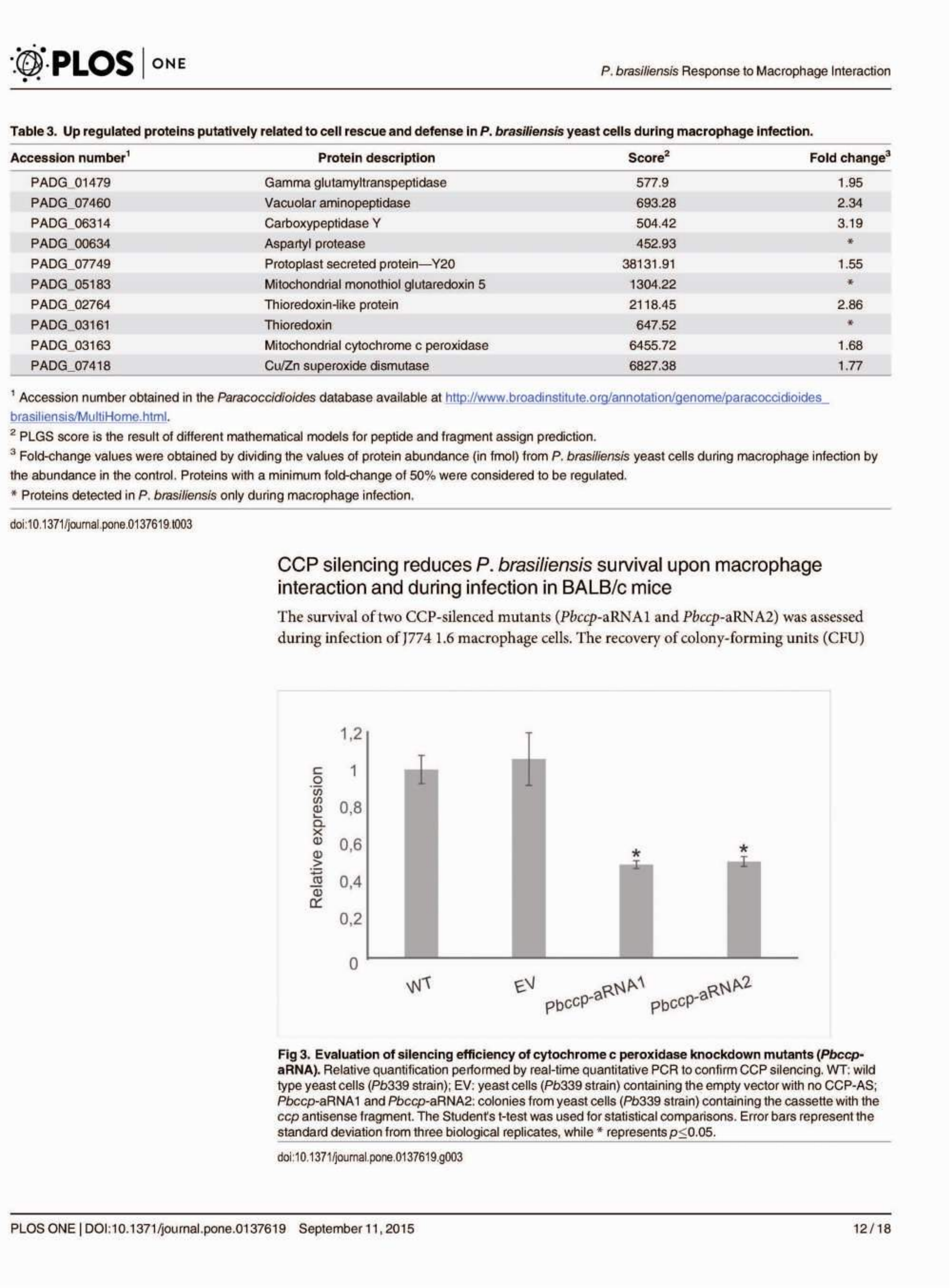

Table 3. Up regulated proteins putatively related to cell rescue and defense in *P. brasiliensis* yeast cells during macrophage infection.

Accession number[1]	Protein description	Score[2]	Fold change[3]
PADG_01479	Gamma glutamyltranspeptidase	577.9	1.95
PADG_07460	Vacuolar aminopeptidase	693.28	2.34
PADG_06314	Carboxypeptidase Y	504.42	3.19
PADG_00634	Aspartyl protease	452.93	*
PADG_07749	Protoplast secreted protein—Y20	38131.91	1.55
PADG_05183	Mitochondrial monothiol glutaredoxin 5	1304.22	*
PADG_02764	Thioredoxin-like protein	2118.45	2.86
PADG_03161	Thioredoxin	647.52	*
PADG_03163	Mitochondrial cytochrome c peroxidase	6455.72	1.68
PADG_07418	Cu/Zn superoxide dismutase	6827.38	1.77

[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] PLGS score is the result of different mathematical models for peptide and fragment assign prediction.

[3] Fold-change values were obtained by dividing the values of protein abundance (in fmol) from *P. brasiliensis* yeast cells during macrophage infection by the abundance in the control. Proteins with a minimum fold-change of 50% were considered to be regulated.

* Proteins detected in *P. brasiliensis* only during macrophage infection.

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CCP silencing reduces *P. brasiliensis* survival upon macrophage interaction and during infection in BALB/c mice

The survival of two CCP-silenced mutants (*Pbccp*-aRNA1 and *Pbccp*-aRNA2) was assessed during infection of J774 1.6 macrophage cells. The recovery of colony-forming units (CFU)

Fig 3. Evaluation of silencing efficiency of cytochrome c peroxidase knockdown mutants (*Pbccp*-aRNA). Relative quantification performed by real-time quantitative PCR to confirm CCP silencing. WT: wild type yeast cells (*Pb*339 strain); EV: yeast cells (*Pb*339 strain) containing the empty vector with no CCP-AS; *Pbccp*-aRNA1 and *Pbccp*-aRNA2: colonies from yeast cells (*Pb*339 strain) containing the cassette with the *ccp* antisense fragment. The Student's t-test was used for statistical comparisons. Error bars represent the standard deviation from three biological replicates, while * represents $p \leq 0.05$.

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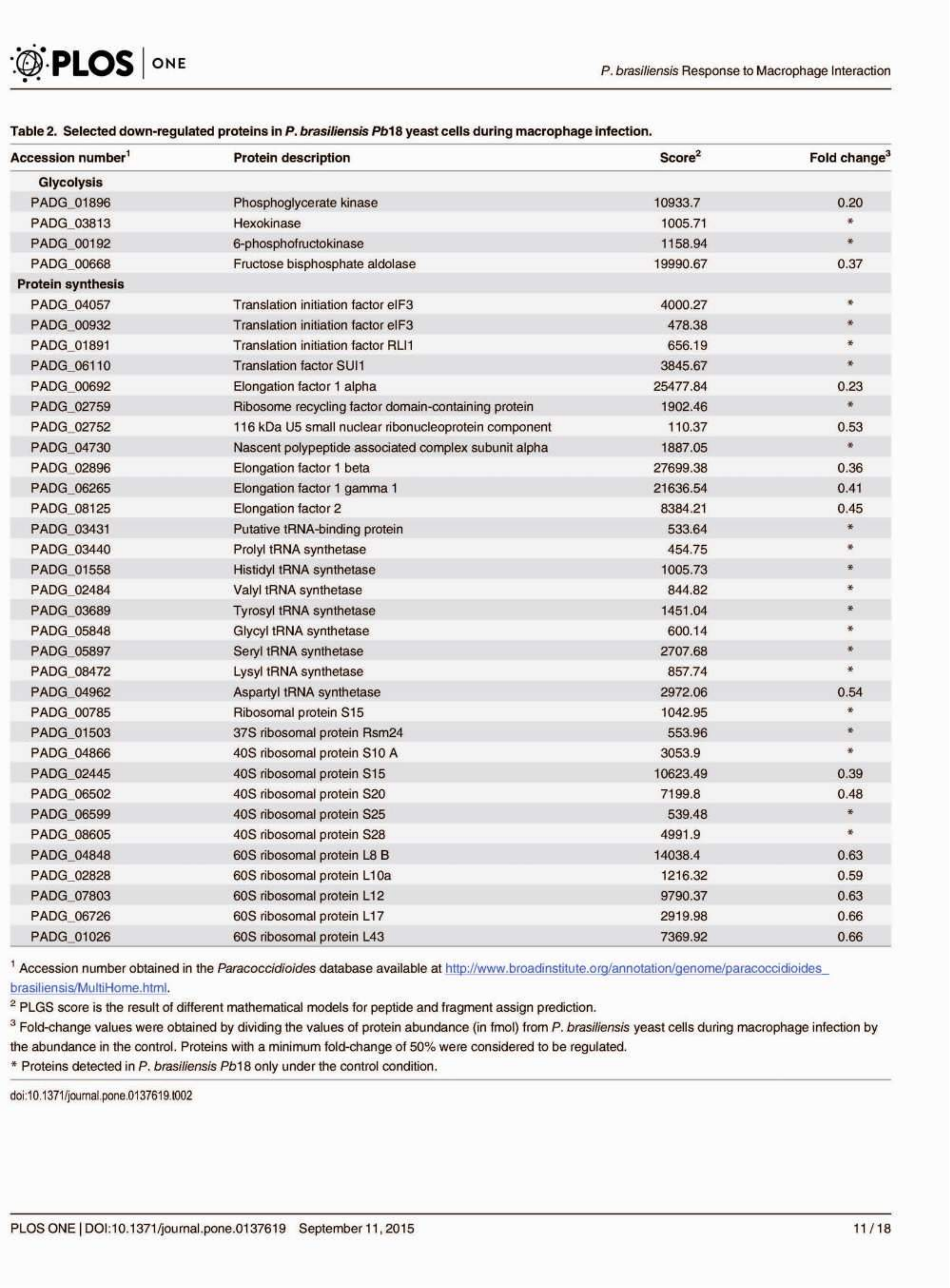

PLOS | ONE *P. brasiliensis* Response to Macrophage Interaction

Table 2. Selected down-regulated proteins in *P. brasiliensis Pb*18 yeast cells during macrophage infection.

Accession number[1]	Protein description	Score[2]	Fold change[3]
Glycolysis			
PADG_01896	Phosphoglycerate kinase	10933.7	0.20
PADG_03813	Hexokinase	1005.71	*
PADG_00192	6-phosphofructokinase	1158.94	*
PADG_00668	Fructose bisphosphate aldolase	19990.67	0.37
Protein synthesis			
PADG_04057	Translation initiation factor eIF3	4000.27	*
PADG_00932	Translation initiation factor eIF3	478.38	*
PADG_01891	Translation initiation factor RLI1	656.19	*
PADG_06110	Translation factor SUI1	3845.67	*
PADG_00692	Elongation factor 1 alpha	25477.84	0.23
PADG_02759	Ribosome recycling factor domain-containing protein	1902.46	*
PADG_02752	116 kDa U5 small nuclear ribonucleoprotein component	110.37	0.53
PADG_04730	Nascent polypeptide associated complex subunit alpha	1887.05	*
PADG_02896	Elongation factor 1 beta	27699.38	0.36
PADG_06265	Elongation factor 1 gamma 1	21636.54	0.41
PADG_08125	Elongation factor 2	8384.21	0.45
PADG_03431	Putative tRNA-binding protein	533.64	*
PADG_03440	Prolyl tRNA synthetase	454.75	*
PADG_01558	Histidyl tRNA synthetase	1005.73	*
PADG_02484	Valyl tRNA synthetase	844.82	*
PADG_03689	Tyrosyl tRNA synthetase	1451.04	*
PADG_05848	Glycyl tRNA synthetase	600.14	*
PADG_05897	Seryl tRNA synthetase	2707.68	*
PADG_08472	Lysyl tRNA synthetase	857.74	*
PADG_04962	Aspartyl tRNA synthetase	2972.06	0.54
PADG_00785	Ribosomal protein S15	1042.95	*
PADG_01503	37S ribosomal protein Rsm24	553.96	*
PADG_04866	40S ribosomal protein S10 A	3053.9	*
PADG_02445	40S ribosomal protein S15	10623.49	0.39
PADG_06502	40S ribosomal protein S20	7199.8	0.48
PADG_06599	40S ribosomal protein S25	539.48	*
PADG_08605	40S ribosomal protein S28	4991.9	*
PADG_04848	60S ribosomal protein L8 B	14038.4	0.63
PADG_02828	60S ribosomal protein L10a	1216.32	0.59
PADG_07803	60S ribosomal protein L12	9790.37	0.63
PADG_06726	60S ribosomal protein L17	2919.98	0.66
PADG_01026	60S ribosomal protein L43	7369.92	0.66

[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] PLGS score is the result of different mathematical models for peptide and fragment assign prediction.

[3] Fold-change values were obtained by dividing the values of protein abundance (in fmol) from *P. brasiliensis* yeast cells during macrophage infection by the abundance in the control. Proteins with a minimum fold-change of 50% were considered to be regulated.

* Proteins detected in *P. brasiliensis Pb*18 only under the control condition.

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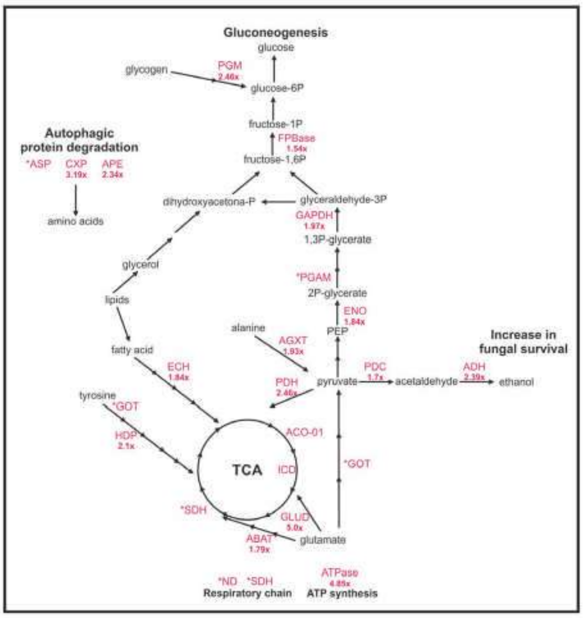

Fig 2. Molecular mechanism used by *P. brasiliensis* to survive inside macrophages. The up regulated enzymes in *P. brasiliensis* during macrophage interaction are: PGM: phosphoglucomutase; PBPase: fructose 1,6-biphosphatase; GAPDH glyceraldehyde 3-phosphate dehydrogenase; PGAM: phosphoglycerate mutase; ENO: enolase; PDC: pyruvate decarboxylase; ADH: alcohol dehydrogenase; PDH: pyruvate dehydrogenase SDH: succinate dehydrogenase, ECH: enoyl-CoA hydratase; AGXT: alanine glyoxylate aminotransferase; GOT: aspartate aminotransferase; HDP: 4-hydroxyphenylpyruvate dioxygenase; ABAT: 4-aminobutyrate aminotransferase; GLUD: glutamate dehydrogenase; ASP: aspartyl protease; CXP: carboxypepetidase Y; APE: vacuolar aminopeptidase; ND: NADH ubiquinone oxidoreductase; and ATPase: ATP synthase. The numbers before enzyme names represent increasing rates in the protein expression during macrophage interaction. The asterisk represents the proteins detected in *P. brasiliensis* only during macrophage infection.

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Therefore, we analyzed the role of the protein cytochrome c peroxidase (CCP) in fungal virulence because the enzyme contributes to the fungal antioxidant defense [36]. A silenced mutant strain for this gene was generated using antisense technology [15,24,37]. The knockdown mutant was obtained in the *Pb*339 strain, which was demonstrated to be the most feasible strain for genetic transformation in our laboratory [37]. The efficiency of the gene silencing from two *Paracoccidioides* transformants was evaluated by qRT-PCR. The silencing efficiency obtained for the CCP knockdown mutants was approximately 50%. The mutant strain obtained with the empty binary vector (EV) depicted no significant difference in the *ccp* expression level when compared with the wild type strain (WT) (Fig 3). To evaluate the sensitivity of the *ccp* silenced strain to oxidative stress, we analyzed the growth of two *P. brasiliensis* isolates (*Pb*18 wild type and *Pb*339 wild type), EV and *Pbccp*-aRNA strains using solid medium supplemented with menadione, that increases mitochondrial-generated ROS stress. The *ccp*-aRNA strains were more sensitive to 40 μM and 80 μM of menadione compared to the wild types isolates and EV strains (Fig 4, Panel B). The strains presented the same *in vitro* growth profile in absence of menadione (Fig 4, panel A). The results strongly suggest that CCP plays a role in avoiding cell damage caused by oxidative stress.

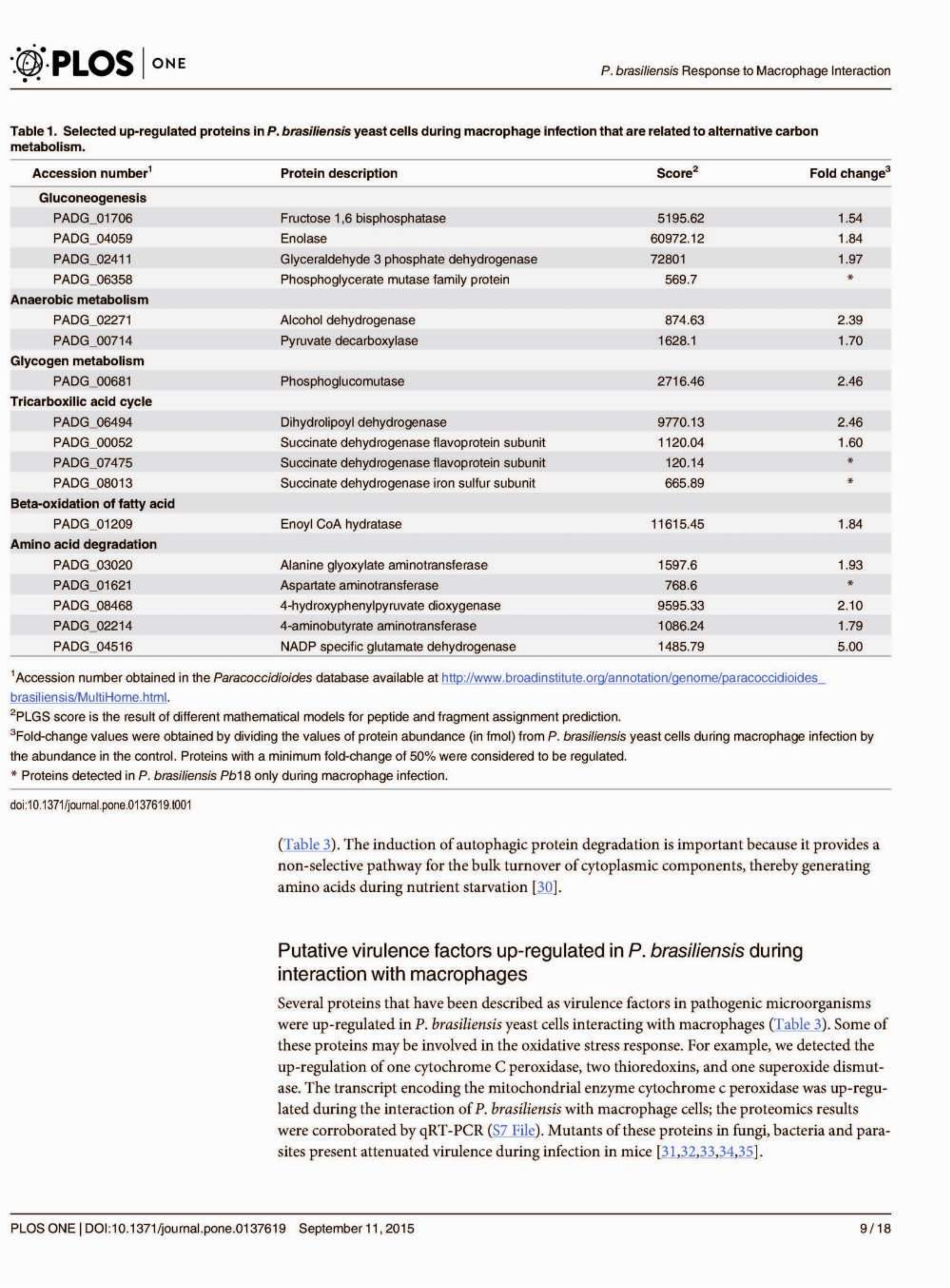

Table 1. Selected up-regulated proteins in *P. brasiliensis* yeast cells during macrophage infection that are related to alternative carbon metabolism.

Accession number[1]	Protein description	Score[2]	Fold change[3]
Gluconeogenesis			
PADG_01706	Fructose 1,6 bisphosphatase	5195.62	1.54
PADG_04059	Enolase	60972.12	1.84
PADG_02411	Glyceraldehyde 3 phosphate dehydrogenase	72801	1.97
PADG_06358	Phosphoglycerate mutase family protein	569.7	*
Anaerobic metabolism			
PADG_02271	Alcohol dehydrogenase	874.63	2.39
PADG_00714	Pyruvate decarboxylase	1628.1	1.70
Glycogen metabolism			
PADG_00681	Phosphoglucomutase	2716.46	2.46
Tricarboxilic acid cycle			
PADG_06494	Dihydrolipoyl dehydrogenase	9770.13	2.46
PADG_00052	Succinate dehydrogenase flavoprotein subunit	1120.04	1.60
PADG_07475	Succinate dehydrogenase flavoprotein subunit	120.14	*
PADG_08013	Succinate dehydrogenase iron sulfur subunit	665.89	*
Beta-oxidation of fatty acid			
PADG_01209	Enoyl CoA hydratase	11615.45	1.84
Amino acid degradation			
PADG_03020	Alanine glyoxylate aminotransferase	1597.6	1.93
PADG_01621	Aspartate aminotransferase	768.6	*
PADG_08468	4-hydroxyphenylpyruvate dioxygenase	9595.33	2.10
PADG_02214	4-aminobutyrate aminotransferase	1086.24	1.79
PADG_04516	NADP specific glutamate dehydrogenase	1485.79	5.00

[1] Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html.

[2] PLGS score is the result of different mathematical models for peptide and fragment assignment prediction.

[3] Fold-change values were obtained by dividing the values of protein abundance (in fmol) from *P. brasiliensis* yeast cells during macrophage infection by the abundance in the control. Proteins with a minimum fold-change of 50% were considered to be regulated.

* Proteins detected in *P. brasiliensis Pb*18 only during macrophage infection.

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(Table 3). The induction of autophagic protein degradation is important because it provides a non-selective pathway for the bulk turnover of cytoplasmic components, thereby generating amino acids during nutrient starvation [30].

Putative virulence factors up-regulated in *P. brasiliensis* during interaction with macrophages

Several proteins that have been described as virulence factors in pathogenic microorganisms were up-regulated in *P. brasiliensis* yeast cells interacting with macrophages (Table 3). Some of these proteins may be involved in the oxidative stress response. For example, we detected the up-regulation of one cytochrome C peroxidase, two thioredoxins, and one superoxide dismutase. The transcript encoding the mitochondrial enzyme cytochrome c peroxidase was up-regulated during the interaction of *P. brasiliensis* with macrophage cells; the proteomics results were corroborated by qRT-PCR (S7 File). Mutants of these proteins in fungi, bacteria and parasites present attenuated virulence during infection in mice [31,32,33,34,35].

and 3,384 in the control cells and those obtained after macrophage infection, respectively). A rate of 57.5% of the identified peptides were obtained from peptide match type data in the first pass and 6.5% from the second pass. A total of 17% of the peptides were identified by a missed trypsin cleavage, and an in-source fragmentation rate of 9% was observed (S3 File).

S4 File depicts the accuracy of the m/z fragment matches in the database. A total of 97.2% of the peptides were assigned with up to 15 ppm m/z of error. S5 File shows the abundant dynamic range of the identified proteins; the distribution of protein concentrations comprised 3 orders of magnitude. A total of 308 differentially expressed proteins were identified in *P. brasiliensis* yeast cells derived from infected macrophages and are depicted in S1 and S2 Files. A fold-change difference in the protein level of 50% in comparison with the control cells was used to identify the regulated proteins. A total of 139 proteins were positively regulated during macrophage infection (S1 File), while 179 proteins were down-regulated (S2 File).

Most of the up-regulated proteins (S1 File, S6 File, panel A) were related to amino acid metabolism (10.9% of the total), cell rescue, defense and virulence (10.9% of the total) and molecules involved in protein synthesis (7.8% of the total).

The most regulated functional classes in the down-regulated proteins included protein synthesis (17.9% of the total) and amino acid metabolism (11.7%). A total of 9.6% of the identified proteins were related to energy production, including enzymes acting in glycolysis, the tricarboxylic acid pathway and the electron transport chain, as well as glyoxylate and methylcitrate cycle components.

S6 File depicts the functional classes of the upregulated proteins (panel A) and down regulated proteins (panel B).

P. brasiliensis adapts to the macrophage milieu by reprogramming its metabolism to produce glucose and inhibiting protein synthesis

The proteomic approach provided new insights into the molecular mechanisms used by *P. brasiliensis* to adapt to the macrophage environment. As depicted in Table 1, fructose1,6-biphosphatase was induced, suggesting an increase in gluconeogenesis to provide glucose. The anaplerotic precursors for glucose are most likely provided by the carbon backbones released by the amino acid degradation pathways (Table 1). Enzymes related to glutamate (glutamate dehydrogenase), alanine (alanine glyoxylate aminotransferase) and aspartate (aspartate aminotransferase) were induced, suggesting their role in production of glucose precursors. Moreover, fatty acids are probably used as fuel for fungal survival inside phagocytes, as suggested by the induction of the enzyme enoyl CoA hydratase. Ethanol production appeared to be increased based on the induction of pyruvate decarboxylase and alcohol dehydrogenase; the pyruvate is most likely provided through amino acid degradation. The increased ethanol production could contribute to fungal survival inside the macrophage because ethanol production contributes to pathogenesis [29]. The up regulated processes induced in *P. brasiliensis* upon internalization by macrophages are depicted in Fig 2.

As depicted in Table 2, the glycolytic-specific enzymes phosphofructokinase 1 and hexokinase were repressed, indicating that glucose was not used as an energy source and reinforcing that *P. brasiliensis* was facing a glucose-poor environment inside the macrophages. Quantification of the transcript encoding fructose 1,6 biphosphatase (*pbpase*) corroborated the proteomics data (S7 File.). The inhibition of protein synthesis in the poor environment inside the macrophages was strongly suggested by the down regulation of a large number of proteins related to that process (S2 File and Table 2).

It is important to highlight the overexpression of proteins related to autophagic protein degradation process, such as vacuolar aminopeptidase, carboxypeptidase Y and aspartyl protease

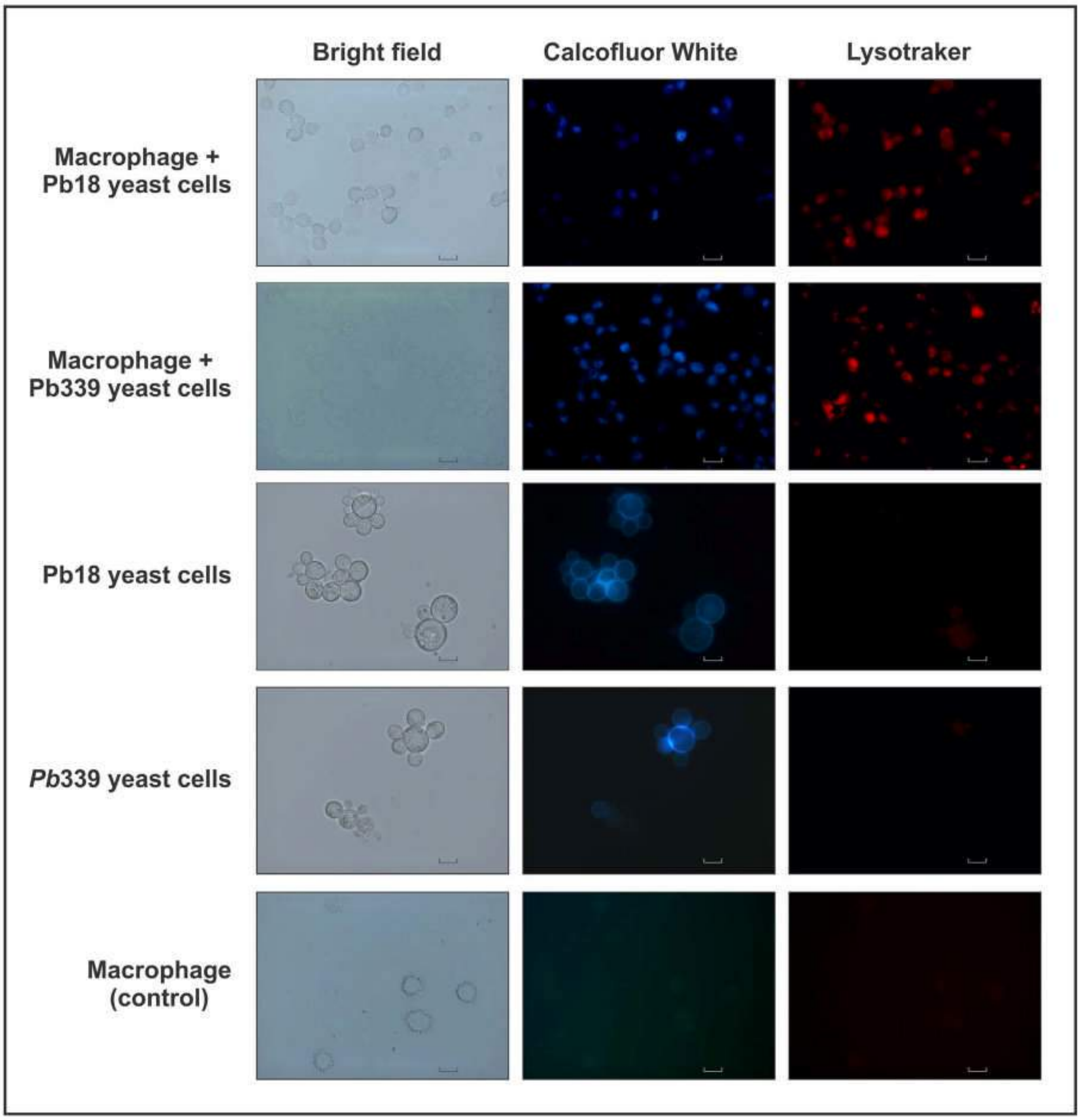

Fig 1. Interaction of *P. brasiliensis* yeast cells with macrophages and evaluation of phagolysosome maturation. The interaction assay was performed using two *P. brasiliensis* isolates (*Pb*18 and *Pb*339) and are shown in the lanes named macrophages + *Pb*18 yeast cells and macrophages + *Pb*339 yeast cells, respectively. The pictures were taken in bright field (shown in the bright field column), at 395/420 nm for Calcofluor probe (shown in the Calcofluor white column) and at 579/599 nm for Lysotracker probe (shown in the Lysotracker column). Fungal control cells are shown in the lanes named *Pb*18 yeast cells and *Pb*339 yeast cells. Control macrophage cells were also performed and are shown in the lane named macrophage (control). All representative pictures were taken using an Axioscope microscope (Carl Zeiss) and magnified 1000X.

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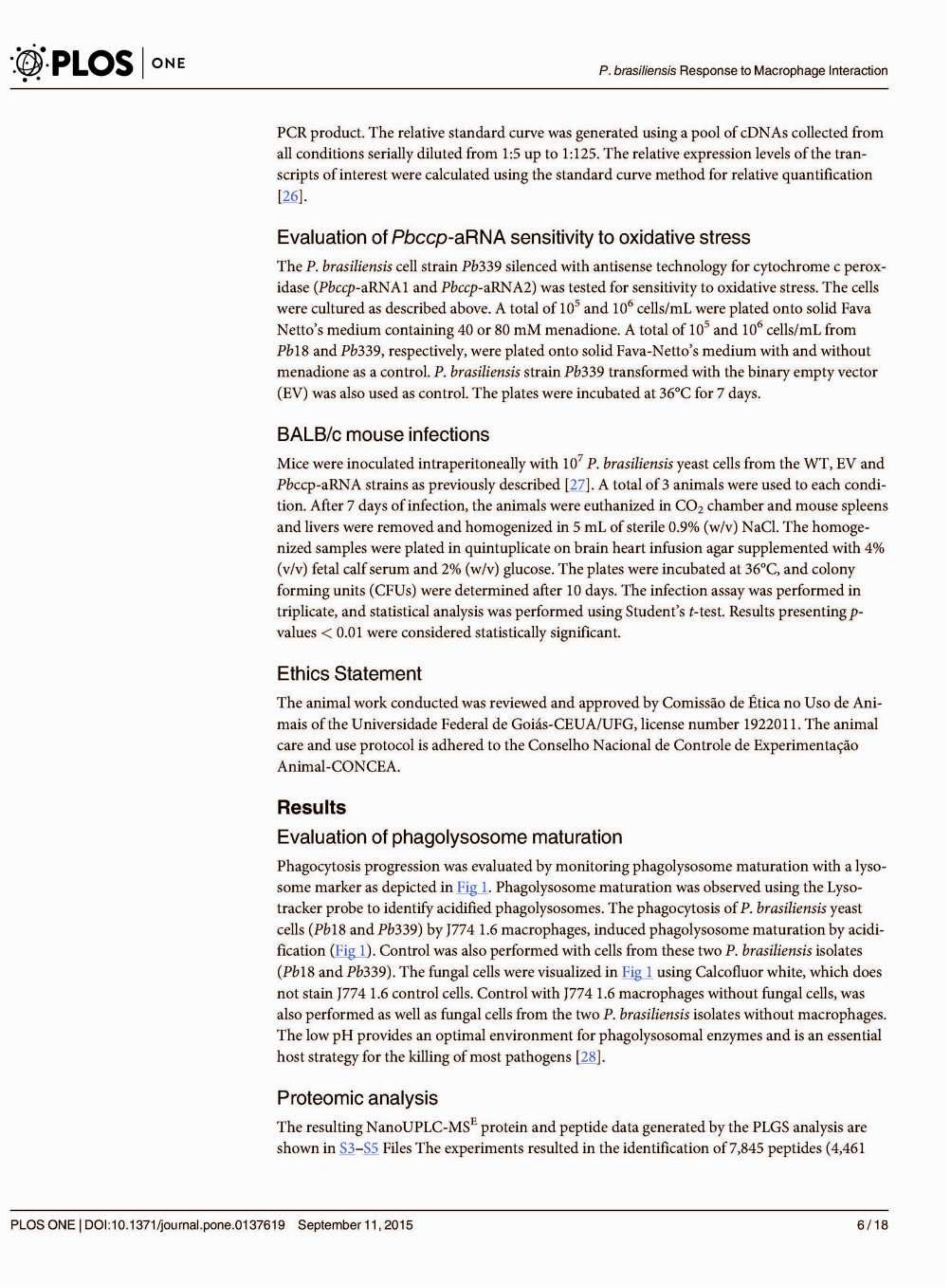

PCR product. The relative standard curve was generated using a pool of cDNAs collected from all conditions serially diluted from 1:5 up to 1:125. The relative expression levels of the transcripts of interest were calculated using the standard curve method for relative quantification [26].

Evaluation of *Pbccp*-aRNA sensitivity to oxidative stress

The *P. brasiliensis* cell strain *Pb*339 silenced with antisense technology for cytochrome c peroxidase (*Pbccp*-aRNA1 and *Pbccp*-aRNA2) was tested for sensitivity to oxidative stress. The cells were cultured as described above. A total of 10^5 and 10^6 cells/mL were plated onto solid Fava Netto's medium containing 40 or 80 mM menadione. A total of 10^5 and 10^6 cells/mL from *Pb*18 and *Pb*339, respectively, were plated onto solid Fava-Netto's medium with and without menadione as a control. *P. brasiliensis* strain *Pb*339 transformed with the binary empty vector (EV) was also used as control. The plates were incubated at 36°C for 7 days.

BALB/c mouse infections

Mice were inoculated intraperitoneally with 10^7 *P. brasiliensis* yeast cells from the WT, EV and *Pb*ccp-aRNA strains as previously described [27]. A total of 3 animals were used to each condition. After 7 days of infection, the animals were euthanized in CO_2 chamber and mouse spleens and livers were removed and homogenized in 5 mL of sterile 0.9% (w/v) NaCl. The homogenized samples were plated in quintuplicate on brain heart infusion agar supplemented with 4% (v/v) fetal calf serum and 2% (w/v) glucose. The plates were incubated at 36°C, and colony forming units (CFUs) were determined after 10 days. The infection assay was performed in triplicate, and statistical analysis was performed using Student's *t*-test. Results presenting *p*-values < 0.01 were considered statistically significant.

Ethics Statement

The animal work conducted was reviewed and approved by Comissão de Ética no Uso de Animais of the Universidade Federal de Goiás-CEUA/UFG, license number 1922011. The animal care and use protocol is adhered to the Conselho Nacional de Controle de Experimentação Animal-CONCEA.

Results

Evaluation of phagolysosome maturation

Phagocytosis progression was evaluated by monitoring phagolysosome maturation with a lysosome marker as depicted in Fig 1. Phagolysosome maturation was observed using the Lysotracker probe to identify acidified phagolysosomes. The phagocytosis of *P. brasiliensis* yeast cells (*Pb*18 and *Pb*339) by J774 1.6 macrophages, induced phagolysosome maturation by acidification (Fig 1). Control was also performed with cells from these two *P. brasiliensis* isolates (*Pb*18 and *Pb*339). The fungal cells were visualized in Fig 1 using Calcofluor white, which does not stain J774 1.6 control cells. Control with J774 1.6 macrophages without fungal cells, was also performed as well as fungal cells from the two *P. brasiliensis* isolates without macrophages. The low pH provides an optimal environment for phagolysosomal enzymes and is an essential host strategy for the killing of most pathogens [28].

Proteomic analysis

The resulting NanoUPLC-MS^E protein and peptide data generated by the PLGS analysis are shown in S3–S5 Files The experiments resulted in the identification of 7,845 peptides (4,461 software from the Waters Corporation [23]. The minimum repeat rate for each protein in all replicates was 2.

Quality parameter such as the dynamic range of the experiments, peptide detection type, and mass accuracy were calculated for each condition using the software MassPivot and Spot-Fire as previously described [21]. Proteins that presented 50% differences in expression values compared to the control were considered to be regulated.

Construction of the cytochrome c peroxidase (CCP) knockdown mutant

The antisense-RNA (aRNA) strategy was used as previously described [24]. Briefly, DNA from *Pb*339 yeast cells was obtained as described [15,25] and used to obtain the aRNA of *Pb*339 cytochrome c peroxidase (*ccp*). The oligonucleotides as*ccp*-sense 5’CCGCTCGAGCGGGATAAGGAAACTGGAACTGGAG 3’ and as*ccp*-antisense, 5’GGCGCGCCCCTGAGAGTGACCACGCTG 3’ were synthesized to amplify aRNA from DNA (PADG_03163; Accession number obtained in the *Paracoccidioides* database available at http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). Plasmid construction, *Agrobacterium tumefaciens* transformation and transformants selection was performed as described [15]. *P. brasiliensis* yeast cells were also transformed with the empty parental vector pUR5750 (EV) as a control for the assays performed in this study. Investigation of *Pbccp* gene expression was performed after consecutive sub culturing by quantitative real-time PCR. The interaction assay of two silenced mutants (*Pb*ccp-aRNA1 and *Pb*ccp-aRNA2) with J774 1.6 cells was performed using a yeast:macrophage ratio of 5:1. The control cells used in this assay were yeast cells from wild type *Pb*339 and *Pb*18 yeast cells transformed with the empty vector in the same yeast:macrophage ratio. The interaction assay using the silenced mutants *Pb*ccp-aRNA1 and *Pb*ccp-aRNA2 and the controls was performed in triplicate. Statistical analysis was performed using Student’s *t*-test. Results presenting p-values < 0.01 were considered statistically significant.

Quantitative real-time PCR (qRT-PCR)

The evaluation of transcriptional levels was performed by qRT-PCR. Total RNA was extracted using Trizol (TRI Reagent, Sigma-Aldrich, St. Louis, MO, USA) and mechanical cell rupture using the Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK, USA). Then, *in vitro* reverse transcription (SuperScript III First-Strand Synthesis SuperMix; Invitrogen, Life Technologies) was performed, and the cDNAs were subjected to qRT-PCR using the SYBR green PCR master mix (Life Technologies, Foster City, CA, USA) in the StepOnePlus real-time PCR system (Life Technologies). The expression values were calculated using the transcript encoding alpha tubulin (XM_002796593) as the endogenous control in the silencing mutation experiment and the transcript encoding beta tubulin (XM_002794440) in the *P. brasiliensis* interaction with macrophage cells experiment. The oligonucleotides used in the qRT-PCR to confirm the proteomic data were: beta tubulin sense: 5’-GATAACGAGGCTCTGTATGATA-3’; beta tubulin atsense: 5’ATGTTGACGGCGAGTTTGCG-3’; pbpase sense: 5’-GCCACTGGTGACTTTACCCT-3’; pbpase atsense: 5’-CATCTCCGGTGGTATTTGCG-3’; ccp RT sense: 5’-CTTTGACGACCGCGAGATTG-3’; and ccp RT atsense: 5’-GACCGTTCCACTTTCTCCAG-3’.

The oligonucleotides used in the CCP silencing mutation experiments were: alpha tubulin sense: 5’-ATGAAACGGCAAATCCCACCA-3’; alpha tubulin antisense 5’- ACAGTGCTTGGGAACTATACC -3’; ccp-sense: 5’ CTTTGACGACCGCGAGATTG 3’ and ccp-antisense 5’ GACCGTTCCACTTTCTCCAG. The qRT-PCR reaction was performed in triplicate for each cDNA sample, and a melting curve analysis was performed to confirm the detection of a single medium. The obtained protein extracts were quantified using the Bradford reagent (Sigma Aldrich) with bovine serum albumin (BSA) (Sigma Aldrich) as the standard.

Digestion of protein extracts and nano-ESI-UPLC-MS^E analyses

A total of 100 μg of each protein extract was used for trypsin digestion as previously described [21,22]. Briefly, 10 μL of 50 mM ammonium bicarbonate buffer, pH 8.5, was added to the samples, which were treated with 0.2% RapiGEST SF Surfactant (v/v) (Waters, Milford, MA, USA) and incubated in a dry bath at 80°C for 15 min. The samples were reduced with 100 mM DTT (GE Healthcare, Piscataway, NJ, USA) at 60°C for 30 min, and alkylated with 300 mM iodacetamide (GE Healthcare, Piscataway, NJ, USA) at room temperature for 30 min. Then, 20 μL of trypsin (50 ng/mL) (Promega, Madison, WI, USA) was added to digest the samples at 37°C in a dry bath for 16 h. To cleave and precipitate the RapiGEST reagent, 20 μL of trifluoroacetic acid (TFA) solution 5% (v/v) was added to the samples, followed by incubation for 90 min at 37°C. The supernatants were dried in a speed vacuum (Eppendorf, Hamburg, Germany) for 5 h. All obtained peptides were suspended in 100 μL of a solution containing 20 mM of ammonium formiate and 200 fmol/μL of PHB (Rabbit Phosphorylase B) (Waters Corporation, Manchester, UK) (MassPREP protein). Nanoscale LC separation of tryptic peptides was performed using a nanoACQUITY system (Waters) equipped with two reverse phase columns working in basic and acidic conditions. The first column was a nanoEase BEH130 C18 (1.7 μm, 100 μm x 100 mm; Waters, USA), and the second was a NanoAcquity UPLC column BEH 130 C18 (1.7 μm, 100 μm × 100 mm; Waters, USA). Mass spectrometry analysis was performed on a Synapt G1 MS (Waters, USA) equipped with a nanoelectronspray source and two mass analyzers: a quadrupole and a time-of-flight (TOF) operating in TOF V-mode. Data were obtained using the instrument in the MS^E mode, which switches the low energy (6 V) and elevated energy (20–40 V) acquisition modes every 0.4 s. Samples were analyzed from three replicates.

Data processing and protein identification

The MS data obtained using the label-free MS^E protocol was processed using the ProteinLynx Global Server (PLGS) version 2.4 (Waters). The data were subjected to automatic background subtraction, deisotoping and charge state deconvolution. After processing, each ion comprised an exact mass-retention time (EMRT) that contained the retention time, intensity-weighted average charge, inferred molecular weight based on charge and *m/z*, and the deconvoluted intensity. Then, the processed spectra were searched against *Paracoccidioides brasiliensis* Pb18 protein sequences (Broad Institute; http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html) together with random sequences. The mass error tolerance for peptide identification was under 50 ppm. The identified proteins showed a minimum of 1 matched peptide and 5 fragments, with at least 2 fragments belonging to the same peptide. Moreover, modifications such as methionine oxidation and serine, threonine and tyrosine phosphorylation were considered. A protein that showed a variance coefficient of 0.08 and that was detected in all replicates was used to normalize the protein expression levels in the samples (accession number: PADG_11272). The comparison of protein abundance was performed based on the average intensity value of the top three ionized tryptic peptides of this internal standard protein and was used to convert the average intensity of the analyte peptides to the corresponding absolute quantity of proteins loaded onto the column. Expression^E informatics v.2.5.2 was used for proper quantitative comparisons. The identified proteins were organized by the expression algorithm, into a statistically significant list, corresponding to induced and reduced regulation ratios between the infected and control groups. The mathematical model used to calculate the ratios was a part of the Expression algorithm inside the PLGS survival of *P. brasiliensis* inside macrophages and affected fungal survival in the liver and spleen of infected mice.

Materials and Methods

Fungal strains and growth conditions

P. brasiliensis isolates *Pb*18 [18] and Pb339 [19] in the yeast form were used in this work. The yeast cells were cultivated in BHI medium, containing 4% glucose (w/v) for 48 h at 36°C under agitation.

Interaction assay of *P. brasiliensis* and J774 1.6 macrophage cells

J774 1.6 macrophages (Rio de Janeiro Cell Bank—BCRJ/ UFRJ, accession number 0273) were used for the phagocytosis assays. The J774 1.6 cells were cultured in RPMI medium containing bovine fetal serum 10% (v/v) and MEM non-essential amino acid solution (Sigma Aldrich, Missouri, USA) at 36°C and 5% CO_2 until completely confluence. The phagocytosis assay was performed in 12-well polypropylene plates (Greinner Bio-One, USA). A total of 10^6 J774 1.6 macrophages were plated per well in RPMI medium containing IFN-γ (1U/mL) (Sigma Aldrich) and incubated for 24 h at 36°C and 5% CO_2 for adherence and activation. Then, the medium was replaced to a fresh RPMI medium containing IFN-γ (1U/mL) and $5x10^6$ Pb18 yeast cells per well were added to the macrophages, resulting in a yeast:macrophage cell ratio of 5:2, since the doubling time for J7741.6 cells is around 20-24h. The cells were incubated for 24 h at 36°C and 5% CO_2. Then, macrophages were lysed with water and fungal cells recovered. The control condition was obtained by incubating $5x10^6$ yeast cells per well in RPMI medium containing IFN-γ (1U/mL) for 24 h at 36°C and 5% CO_2.

Evaluation of phagolysosome maturation

The maturation of phagolysosomes was assessed using the Lysotracker probe red DND99 (Life Technologies Carlsbad, USA) according to the manufacturer's instructions. Briefly, the macrophage cells were labeled with 75 nM of the Lysotracker probe for 60 min, prior to fungal infection, to avoid labeling of *P. brasiliensis* cells. The interaction assay of J774 1.6 macrophages with *P. brasiliensis* was performed as described, in a 6-well polypropylene plate, with cover slip. After, the cells were washed three times with sterile phosphate buffered saline (PBS), and incubated with 2 mg/mL of fluorescent brightener (Calcofluor white M2R, Sigma Aldrich) for 1 h, to label the fungal cells. Macrophage J774 1.6 cells were used as the control. The cover slips were fixed with 4% paraformaldehyde (Sigma Aldrich) for 1 h, washed three times with sterile PBS removed and photographed at bright field, at 579/599 nm for Lysotracker and at 395/420 nm for Calcofluor White, using an Axioscope A1 fluorescence microscope (Carl Zeiss).

Preparation of protein extracts

The interaction assay of *P. brasiliensis* yeast cells with macrophages was performed as described above. Then, the cells were washed three times with PBS and the macrophages were lysed by the addition of sterile water. The lysates were centrifuged at 8,000 x g for 10 min. The obtained pellet containing *P. brasiliensis* yeast cells was washed three times with water. The pellet was ressuspended in a solution containing 20 mM Tris-HCl, pH 8.8, and 2 mM $CaCl_2$ [20], and the protein extraction was performed in BeadBeater equipment (BioSpec, Bartlesville, USA) in tubes containing 200–500 μm of acid-washed glass beads (Sigma Aldrich) in equal volume of fungal pellet. Control cells were obtained by incubating *P. brasiliensis* yeast cells in RPMI

and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Paracoccidioidomycosis (PCM) is a human systemic mycosis that is restricted to Latin America, particularly Brazil, Colombia and Venezuela [1]. The disease is caused by members of the *Paracoccidioides* genus. These fungi are thermo-dimorphic species that grow as mycelium under saprobic conditions at 22–26°C, or as pathogenic yeast at 36°C [2]. The saprobic form lives in the soil and reaches the lung alveoli upon the inhalation of spores or mycelia fragments by the host, where they interact with epithelial cells and alveolar macrophages [3]. The fungus converts to the yeast pathogenic form at body temperature [4].

Macrophages constitute one of the primary defense mechanisms against infection by *P. brasiliensis;* thus, PCM is considered to be a classic granulomatous disease [5,6]. As a facultative intracellular pathogen, *P. brasiliensis* can persist inside macrophages. Microscopic studies showed that *P. brasiliensis* multiplied intracellularly in macrophages and this could be a factor in pathogenicity [5,6,7,8].

A characteristic of macrophages is the production of copious amounts of oxidants during the respiratory burst process [9]. The oxidative burst, a reaction characterized by increased oxygen uptake and ROS (reactive oxygen species) production, challenges parasite viability [10]. The most important ROS and RNS (reactive nitrogen species) generated inside the phagolysosome are nitric oxide (NO•), peroxynitrite (ONOO-), superoxide anion radical (O2-•), and hydroxyl radical (•OH) [11]. In particular, the success of pathogens is based on their resistance to nitrosative and oxidative stresses, and other environmental attacks [12]. During the infection process, *Paracoccidioides* spp. can cope with the RNS and ROS generated during the respiratory burst of phagocytic cells, as suggested by the arsenal of related transcripts [13]. Indeed, members of the *Paracoccidioides* spp. complex express a powerful antioxidant defense system in the presence of ROS-mediated oxidative stress [14]. Proteomic analysis demonstrated that the fungus presented a global activation of antioxidant enzymes, such as catalases, superoxide dismutases, cytochrome c peroxidase and thioredoxin when exposed to H_2O_2. The activation of the pentose phosphate pathway, a great source of cellular reducing power in the form of NADPH, suggested that there was a shift in the metabolism of yeast cells [14]. Response to nitrosative stress was also evaluated in *Paracoccidioides* sp. [15]. In this sense, a RNA approach to silence the gene encoding cytochrome c peroxidase depicted mutants highly sensitive to nitrosative stress [15]. Enzymes acting in the oxidative stress response also played a role in the nitrosative stress. Additionally, we demonstrated that carbon starvation exerted a strong effect on *Paracoccidioides* sp.. This stress, which is presumably similar to that found in the macrophage environment, evoked a shift to a starvation mode as determined at the transcriptional and proteomic levels. The metabolic alterations included an increase in gluconeogenesis and fermentative ethanol production, activation of fatty acids and amino acid degradation; these strategies are likely used by the pathogen to persist under this type of stress [16].

As described above, studies have begun to elucidate the complex transcriptional and translational programs that *Paracoccidioides* spp. use to survive when exposed to host-like conditions [14,15,16]. Tavares and co-workers (2007) showed that *P. brasiliensis* regulated the expression of 119 classified genes during phagocytosis; these genes were primarily associated with glucose and amino acid limitation, cell wall construction and oxidative stress [17].

One method for analyzing the *P. brasiliensis*-macrophage encounter should be the identification of alterations in the proteome, as the fungus is undergoing phagocytosis. In this way, in the present study, we assessed the response of *P. brasiliensis* to macrophage phagocytosis by employing high throughput proteomic analysis. The importance of the regulated proteins for the survival of *P. brasiliensis* within macrophages was inferred. This study demonstrated that the knockdown of cytochrome c peroxidase resulted in decreased

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