UNIVERSIDADE FEDERAL DE GOIÁS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

# TESE DE DOUTORADO

## Análise proteômica do fungo *Paracoccidioides brasiliensis* sob privação de cobre

**GUILHERME PETITO**

**Goiânia-Goiás**
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UNIVERSIDADE FEDERAL DE GOIÁS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS

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Universidade Federal de Goiás
Instituto de Ciências Biológicas
Programa de Pós-graduação em Genética e Biologia Molecular

Guilherme Petito

# Análise proteômica do fungo *Paracoccidioides brasiliensis* sob privação de cobre

Tese apresentada ao programa de Pós-graduação em Genética e Biologia Molecular (PGBM) da Universidade Federal de Goiás (UFG) para obtenção do título de doutor em Genética e Biologia Molecular.

**Orientadora:** Drª. Célia Maria de Almeida Soares

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Ata Nº **25** da sessão de Defesa de Tese de **Guilherme Petito** que confere o título de Doutor(a) em **Genética e Biologia Molecular.**

Ao/s **vinte e sete dias do mês de abril de 2020**, a partir da(s) **14h**, **por videoconferência, seguindo portaria CAPES no. 36 de 16 de março de 2020 e recomendação da UFG**, realizou-se a sessão pública de Defesa de Tese intitulada "Análise proteômica do fungo *Paracoccidioides brasiliensis* sob privação de cobre". Os trabalhos foram instalados pelo(a) Orientador(a), Professor(a) Doutor(a) **Célia Maria de Almeida Soares (UFG)** com a participação dos demais membros da Banca Examinadora: Professor(a) Doutor(a) **Agenor de Castro Moreira dos Santos Junior (HEMOCENTRO)**, membro titular externo; Professor(a) Doutor(a) **Juliana Santana de Curcio**, membro titular externo; Professor(a) Doutor(a) **Livia do Carmo Silva**, membro titular externo; Professor(a) Doutor(a) **Kleber Santiago Freitas e Silva**, membro titular externo. Durante a argüição os membros da banca **não fizeram** sugestão de alteração do título do **trabalho**. A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Tese tendo sido(a) o(a) candidato(a) **aprovado(a)** pelos seus membros. Proclamados os resultados pelo(a) Professor(a) Doutor(a) **Célia Maria de Almeida Soares**, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, ao(s) **vinte e sete dias do mês de abril de 2020**.

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# Sumário

## Lista de figuras e tabelas

### Capítulo 1



### Capítulo 2

## Símbolos, Siglas e Abreviaturas

| | |
|---|---|
| iTRAQ | Isobariq tag for relative and absolute quantitation |
| Aoxp | Proteína alternativa oxidase |
| ATP7A | P-type copper ATPase pump |
| Atx1p | Chaperona de cobre |
| Ctrp | Proteína transportadora de cobre |
| $Cu^{+}$ | Íon cobre 1+ (Íon cuproso) |
| $Cu^{2+}$ | Íon cobre 2+ (Íon cúprico) |
| DNA | Ácido desoxiribonucleico |
| EBP | *Estradiol Binding Protein - Proteína de Ligação ao Estradiol* |
| GST | Glutationa-S-transferase |
| IFN-γ | Interferon-gama |
| IL-10 | Interleucina 10 |
| Mb | Mega bases |
| mg | Miligrama |
| mL | Mililitro |
| µg | Micrograma |
| µL | Microlitro |
| µM | Micromolar |
| *Pb*01 | Isolado 01 de *Paracoccidioides lutzii* |
| *Pb*18 | Isolado 18 de *Paracoccidioides brasiliensis* |
| *Pb*339 | Isolado 339 de *Paracoccidioides brasiliensis* |
| *Pb*EPM83 | Isolado EM83 *de Paracoccidioides restrepiensis* |
| PCM | Paracoccidioidomicose |
| pH | Potencial hidrogeniônico |
| PS1 | *phylogenetic species 1* |
| PS2 | *phylogenetic species 2* - Espécie filogenética 2 |
| PS3 | *phylogenetic species 3* - Espécie filogenética 3 |
| PS4 | *phylogenetic species 4* - Espécie filogenética 4 |
| qRT-PCR | *Quantitative real-time polymerase chain reaction* - PCR em tempo real |
| RNA | Ácido ribonucleico |
| S1 | Species 1 - Espécie 1 |
| Sod1p | Proteína superóxido dismutase Cu/Zn dependente |
| Sod2p | Proteína superóxido dismutase Mn/Fe dependente |
| Trxp | Proteina tioredoxina |

**Resumo**

O cobre é um importante micronutriente que atua como cofator em enzimas que participam em processos de obtenção de energia, redução de espécies reativas de oxigênio e captação de metal, como o ferro. Estudos com diferentes fungos patogênicos demonstram a importância de um sistema eficiente de homeostase de cobre, bem como o impacto da privação desse metal no metabolismo desses organismos. Entretanto, pouco se sabe acerca do impacto da privação de cobre em fungos patogênicos do gênero *Paracoccidioides*. Dessa forma, objetivou-se com este estudo analisar o comportamento proteômico do fungo *Paracoccidioides brasiliensis* durante a privação de cobre, identificando as alterações metabólicas frente a essa condição. A análise proteômicas foi realizada utilizando espectrometria de massas e marcação das amostras com iTRAQ (Isobariq tag for relative and absolute quantitation). Além disso, foram aplicadas neste estudo metodologias como qRT-PCR, microscopia de fluorescência, atividades enzimáticas e Western bloting, com intuito de validar os dados proteômicos analisados. Foram identificadas 183 proteínas diferencialmente expressas. Proteínas relacionadas à gliconeogênese, beta-oxidação e remodelamento de parede estavam com expressão aumentada na condição de privação de cobre. Identificamos diminuição na expressão de uma importante enzima de detoxificação, a superóxido dismutase Cu/Zn dependente (Sod1p). Por outro lado, as enzimas tioredoxina (Trxp), glutationa-S-transferase (GST) e superóxido dismutase Mn/Fe dependente (Sod2p) estavam up-reguladas. Também enzimas da via das pentoses-fosfato relacionadas à produção de NADPH, importante agente redudor de enzimas de detoxificação foram aumentadas. Uma oxidase alternativa (Aoxp) estava com expressão aumentada, enquanto testes de atividades enzimáticas demonstraram que a atividade da enzima citocromo c oxidase (Coxp) estava diminuída. A expressão  chaperona de cobre, Atx1, relacionada com captação de ferro e descrita como importante em reposta ao estresse oxidativo, estava aumentada. Propomos aqui uma visão geral que apresenta as principais mudanças metabólicas em *P. brasiliensis* sob privação de cobre, que incluem mudanças no processo de obtenção de energia, no fluxo do metabolismo de carbono, no remodelamento de parede, nos processos de detoxificação e de respiração.

**Pavavras-chave:** *Paracoccidioides spp*.; Proteomica; iTRAQ; Paracoccidiodomicose.

**Abstract**

Copper is an important micronutrient that acts as a cofactor in important enzymes that participate in processes of obtaining energy, reducing reactive oxygen species and metal uptake such as iron. Studies with different pathogenic fungi demonstrate the importance of an efficient copper homeostasis system, as well as the impact of this metal deprivation on the metabolism of these organisms. However, little is known about the impact of copper deprivation on pathogenic fungi of the genus *Paracoccidioides.* Thus, the objective of this study was to analyze the proteomic behavior of the fungus *Paracoccidioides brasiliensis* during copper deprivation, identifying metabolic changes in face of this condition. Proteomic analysis was performed using mass spectrometry and labelling the samples with iTRAQ (Isobariq tag for relative and absolute quantitation). In addition, methodologies such as qRT-PCR, fluorescence microscopy, enzymatic activities and Western bloting were applied in this study in order to support the proteomic data analyzed. We identified 183 differentially expressed proteins. Proteins related to gluconeogenesis, beta-oxidation and cell wall remodeling were up-regulated. We identified decreased expression of an important detoxification enzyme, Cu / Zn dependent Superoxide Dismutase (Sod1p). On the other hand, the enzymes Thioredoxin (Trxp), Glutathione S-transferase (GST) and Mn / Fe-dependent superoxide dismutase (Sod2p) were up-regulated. Also NADPH production-related to the pentose-phosphate pathway enzymes. detoxifying enzyme reducing agent. An alternative oxidase (Aoxp) was up-regulated, while enzyme activity tests showed that the activity of the enzyme Cytochrome c oxidase (Coxp) was decreased. An iron uptake-related copper chaperone, Atx1 and described as important in response to oxidative stress, was up-regulated. We propose here an overview that presents the main metabolic changes in *P. brasiliensis* under copper deprivation and that include changes in the energy acquisition process, carbon metabolism flow, wall modeling, detoxification and respiration processes.

**Keywords:** *Paracoccidioides spp* .; Proteomic; iTRAQ; Paracoccidioidomycosis.

# Capítulo I

# 1. Introdução

## 1.1. *Paracoccidioides* spp.

Os fungos são seres vivos amplamente distribuídos pelo ecossistema, sendo encontrados em diferentes regiões e climas, presentes no solo, vegetações e em animais. Possuem grande capacidade de adaptação e sobrevivência. São fundamentais no processo de decomposição da matéria orgânica. Apesar da diversidade de espécies, apenas uma quantidade limitada de fungos é capaz de causar doenças, variando de infecções assintomáticas até letais (MURRAY; ROSENTHAL; PFALLER, 2015).

O complexo *Paracoccidioides* compreende fungos termodimórficos, da família Ajellomycetaceae, ordem dos Onygenales (SALGADO-SALAZAR et al., 2010). Nesta família também estão incluídos os gêneros *Blastomyces, Emmonsia e Histoplasma.* Vivem saprofiticamente na natureza e seu habitat parece ser o solo, em locais de temperaturas moderadas, em regiões úmidas com presença de rios e florestas. Fatores como clima e características do solo influenciam na presença do fungo em determinadas regiões (MARTINEZ, 2017; RESTREPO; MCEWEN; CASTAÑEDA, 2001). *Paracoccidioides spp*. são prevalentes em países da América Latina, com grande distribuição no Brasil, Venezuela, Colômbia e Argentina (BOCCA et al., 2013; RICHINI-PEREIRA et al., 2009; TEIXEIRA et al., 2014).

*Paracoccidioides spp.* crescem, em condições saprófitas ou quando cultivados em temperatura ambiente (18-25 ºC), na forma de micélio. No tecido do hospedeiro ou quando cultivado à 37º C, crescem na forma leveduriforme (FRANCO, 1987; RESTREPO, 1985). Esta mudança na morfologia a partir de alteração na temperatura caracteriza o termodimorfismo. A figura 1 mostra o processo de transição do fungo *Paracoccidioides brasiliensis* da forma de micélio para levedura a partir da mudança na temperatura de cultivo (OLIVEIRA et al., 2017). A forma miceliana é caracterizada por um aglomerado de hifas finas, septadas com clamidoconídios intercalares e terminais, artroconídeos e/ou aleuroconídeos, com aspecto algodonoso e branco. A forma leveduriforme possui a superfície rugosa, apresentando, microscopicamente, células multinucleadas, com brotamentos múltiplos e aspecto semelhante a uma "roda de leme" (Figura 2) (MURRAY; ROSENTHAL; PFALLER, 2015; QUEIROZ-TELLES; ESCUISSATO, 2011).

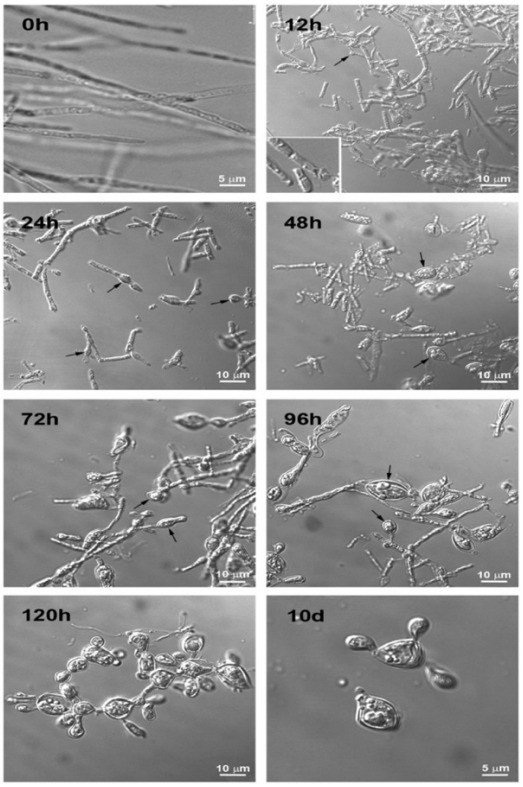


**Figura 1 –** Transição do fungo *Paracoccidioides brasiliensis* da forma de micélio para levedura, após aumento de temperatura de 26ºC para 37ºC.
Fonte: (OLIVEIRA et al., 2017)

A partir de estudos realizados com isolados de diferentes regiões, distintas espécies filogenéticas de *Paracoccidioides* foram descritas, sendo proposto uma classificação filogenética assim distribuída: S1, PS2, PS3 e PS4, além da espécie *Paracoccidioides lutzii* (BOCCA et al., 2013; MATUTE et al., 2006; TEIXEIRA et al., 2014).

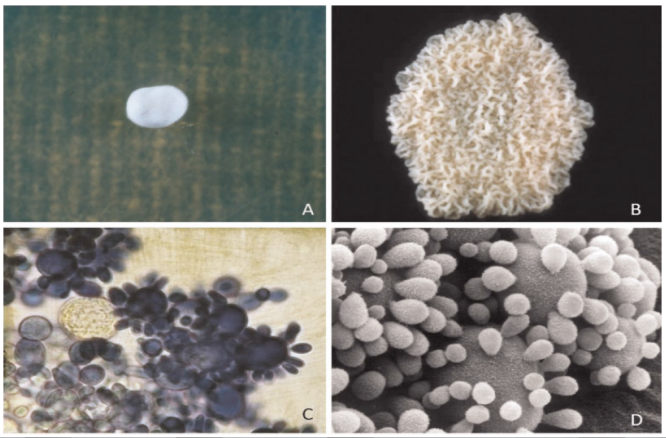


**Figura 2 -** (A) *Paracoccidioides brasiliensis* na fase de micélio. (B) Fase de levedura a 37º C. (C-D) Multibrotamentos de células de levedura, aspecto de "leme de navio", apresentado durante a fase de levedura. Adaptado de: (QUEIROZ-TELLES; ESCUISSATO, 2011).

Recentemente, um estudo propôs que sejam adotados os nomes *Paracoccidioides americana* para PS2, *Paracoccidioides restrepiensis* para PS3, *Paracoccidioides venezuelensis* para PS4 e restringir o uso de *Paracoccidioides brasiliensis* para S1 (TURISSINI et al., 2017). A Figura 3 apresenta as distribuições nas diferentes regiões onde *Paracoccidioides spp.* podem ser encontrados. *Paracoccidioides brasiliensis*, está distribuído no Brasil, Argentina, Paraguai, Peru e Venezuela. *Paracoccidioides americana* é encontrado no Brasil, nos estados de Minas Gerais e São Paulo e na Venezuela. *Paracoccidioides restrepiensis* tem sido descrito como predominantemente restrito à Colômbia. *Paracoccidioides venezuelensis* com prevalência na Venezuela e *Paracoccidioides lutzii* é descrito como endêmico na região Centro-Oeste, principalmente nos estados de Goiás e Mato Grosso (TEIXEIRA et al., 2014; TURISSINI et al., 2017).

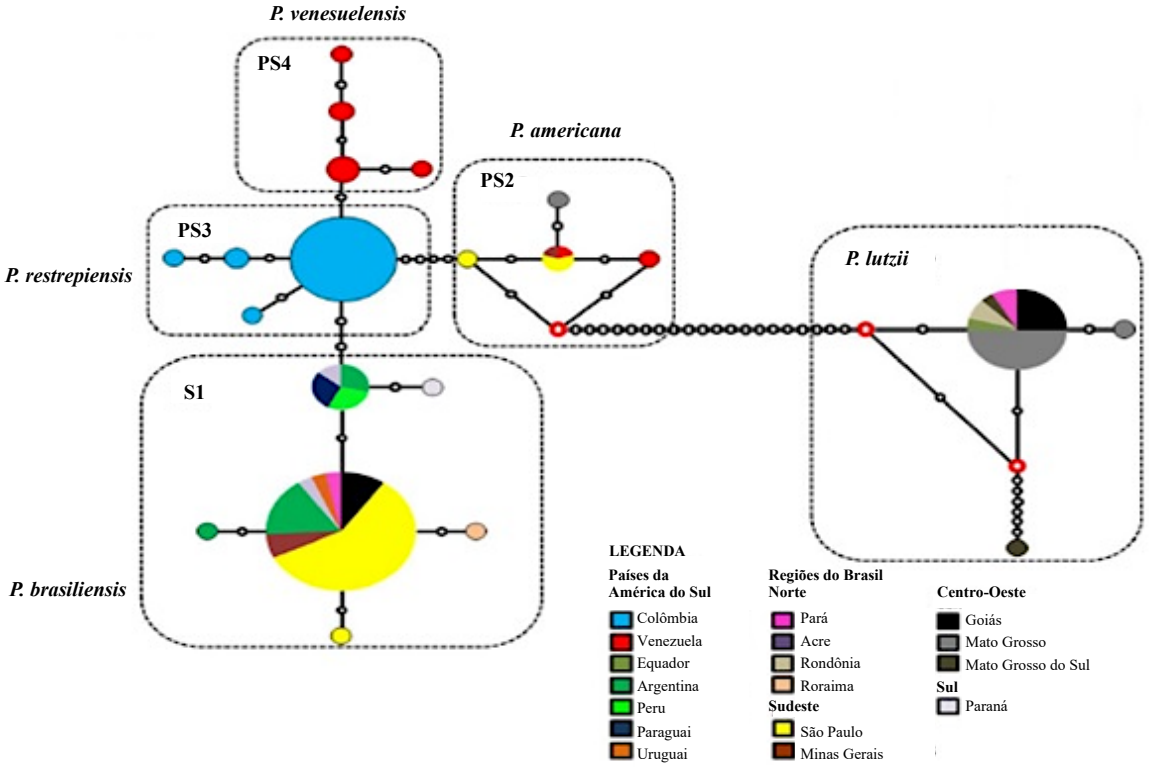


**Figura 3.** Representação das famílias filogenéticas de *Paracoccidioides spp.* e suas distribuições por região. O tamanho da circunferência é proporcional à frequência de cada grupo. As cores indicam a localização mais comum, de acordo com a legenda. Adaptado de: (TEIXEIRA et al., 2014).

Estas diferentes espécies filogenéticas possuem diferentes características morfológicas e moleculares que podem impactar em uma maior ou menor capacidade de infecção. Pigosso e colaboradores (2014), através de estudo proteômico, sugerem o uso de um metabolismo mais anaeróbico para produção de energia, pelo isolado *Pb*01

(*Paracoccidioides lutzii*) em relação aos isolados *Pb*02 (*Paracoccidioides americana*) , *Pb*339 (*Paracoccidioides brasiliensis*) e *Pb*EPM83 (*Paracoccidioides restrepiensis*). Diferenças entre os genomas de *Paracoccidioides spp.* também são descritas. Em um estudo, no qual foi avaliado características do genoma de três isolados (*Pb*03, *P*b18 e *P. lutzii),* foi descrito em seus resultados uma diferença no tamanho do genoma de *Pb*03 (29,1Mb), *Pb*18 (30 Mb) e *P. lutzii* (32 Mb). Uma diferença na quantidade de genes também é descrita, variando de 7.875 no *Pb*03 para 9.132 no *P. lutzii* (DESJARDINS et al., 2011).

### 1.2.Paracoccidioidomicose (PCM)

A paracoccidioidomicose (PCM) é considerada a micose sistêmica endêmica de maior prevalência na América Latina, acometendo principalmente trabalhadores da zona rural, entre 30 e 50 anos de idade, do sexo masculino, podendo levar à morte. A PCM foi descrita pela primeira vez em 1908 por Adolf Lutz, a partir do isolamento do fungo de lesões orais e linfomas cervicais (ABREU E SILVA et al., 2013). O contágio ocorre por meio da inalação de propágulos que alcançam os pulmões. Logo após a inalação, o fungo interaje com macrófagos, importante linha de defesa do hospedeiro, iniciando o processo de sobrevivência e proliferação . Estabelecida a infecção, o fungo pode se difundir por diferentes órgãos e tecidos, por meio de vias hematogênicas ou linfáticas (FRANCO, 1987).

A doença pode se manifestar sob a forma clínica aguda/subaguda (juvenil), mais prevalente em crianças, adolescentes e adultos jovens, de ambos os sexos. É caracterizada por ser de rápida evolução e disseminação para diferentes órgãos, sendo que, geralmente, os pacientes são diagnosticados algumas semanas após os primeiros sintomas. Os pacientes podem apresentar manifestações digestivas, lesões cutâneas, hepatoespenomegalia, linfoadenomegalia e, raramente, problemas pulmonares (Figura 4), Também ocorre a forma crônica (adulta), mais comum e com maior prevalência em adultos com idade entre 30 e 60 anos, a qual compreende a maioria dos casos de PCM diagnosticados (74% a 96%). Neste quadro clinico a doença se inicia de forma lenta e os sintomas pode persistir por meses ou anos. Na maioria dos casos há comprometimento pulmonar, seguido por lesões da mucosa superior oral e a pele (Figura 5) (SHIKANAI-YASUDA et al., 2006, 2017).

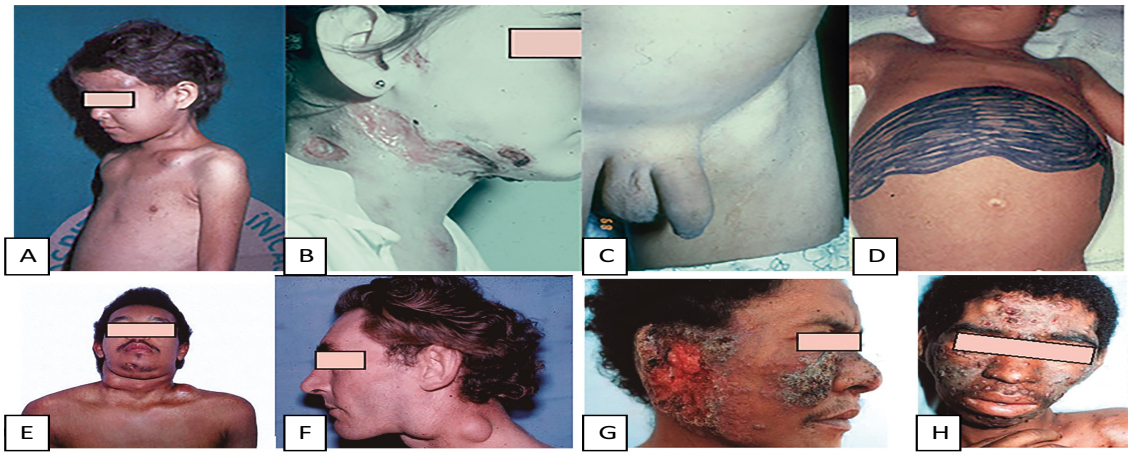


**Figura 4** - Forma aguda/subaguda (juvenil) de PCM. A. Abscessos nas regiões frontal e clavicular. B. Abscesso linfático. C. Linfoadenomegalia inguinal. D. Hepatoesplenomegalia. E. Massa ganglionar na região supraclavicular, cervical e submandibular. F. Linfadenomegalia. G-H. Lesão ulcerativas. Adaptado de (SHIKANAI-YASUDA et al., 2017).

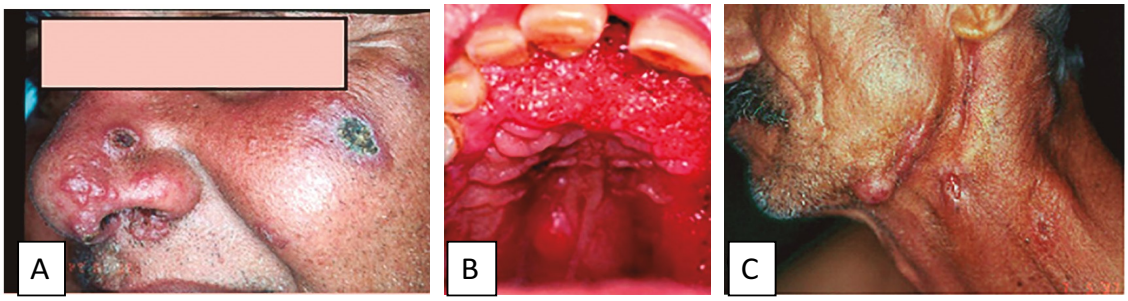


**Figura 5** - Forma crônica de PCM. A. Lesões ulcerativas papulonodulares no rosto. B. Estomatite Moriforme de Aguiar-Pupo. C. Nódulos linfáticos cervical e submandibular fistulado. Adaptado de (SHIKANAI-YASUDA et al., 2017).

Em grande parte dos casos, a infecção a nível pulmonar é assintomática. Assim, um indivíduo pode permanecer com a infecção por longos períodos sem que haja algum tipo de manifestação que indique a presença do fungo no organismo (BUCCHERI et al., 2016; MARTINEZ, 2017). Além disso, quando ocorrem alguns sintomas, geralmente são semelhantes aos causados por infecções bacterianas ou virais. Este fato contribui para o tratamento tardio, uma vez que o paciente ignora ou inicia um tratamento ineficaz para a infecção fúngica (QUEIROZ-TELLES; ESCUISSATO, 2011).

Cerca de 80% dos pacientes com PCM estão no Brasil. Por não ser uma doença de notificação compulsória, dados epidemiológicos são imprecisos neste país. Entre os anos de 2002 e 2004, um total de 175 mortes por ano foram registrados no Brasil em pacientes diagnosticados com PCM (PRADO et al., 2009). Estima-se que a taxa de incidência anual da doença no Brasil varie entre 30 e 40 casos por milhão de habitantes

(BITTENCOURT; DE OLIVEIRA; COUTINHO, 2005; SANTO, 2008; SHANKAR et al., 2011).

Uma maior prevalência da PCM é comumente descrita em indivíduos do gênero masculino, pela interação do hormônio feminino 17-β-estradiol com uma proteína produzida pelo fungo, a *Estradiol Binding Protein* (EBP). Estudos sugerem que esta interação tenha a capacidade de inibir a transição do fungo de micélio para levedura, atenuando sua virulência (ABREU E SILVA et al., 2013). Ainda, durante a fase reprodutiva, sugere-se que mulheres desencadeiam uma resposta imunológica diferente em relação aos homens frente à infecção pelo *Paracoccidioides spp.*. Um estudo realizado com camundongos machos castrados, tratados com estradiol, revelou que estas cobaias produziram níveis elevados de IFN-γ, importante fator de ativação de macrófagos, e menores IL-10, que por sua vez possui ação inibitória sobre macrófagos ativados (PINZAN et al., 2010).

A capacidade de infecção do fungo *Paracoccidioides spp.* está intimamente relacionado com a capacidade de obtenção de elementos essenciais, como íons metálicos, em ambientes inóspitos como no hospedeiro. O fungo desenvolve estratégias que envolvem a modulação na expressão de proteínas e regulação de vias metabólicas fundamentais para sua sobrevivência (DE OLIVEIRA et al., 2014). Neste contexto, o cobre desempenha um papel fundamental para na adaptação, sobrevivência e virulência do fungo durante a interação com hospedeiro (MARCOS et al., 2014).

### 1.3. A importância do cobre para *Paracoccidioides spp.*

A capacidade redox do cobre torna este metal um cofator essencial para diversas enzimas que são críticas para o crescimento celular, virulência e sobrevivência do fungo (SAMANOVIC et al., 2012). O cobre em sua forma reduzida ($cu^{1+}$) desempenha um papel importante em processos celulares (KIM; NEVITT; THIELE, 2008). Por outro lado, assim como o ferro, o excesso de cobre pode ser tóxico para a célula, por envolver-se com reações químicas com o $H_2O_2$, gerando radicais livres, em um processo conhecido como reação de Fenton-Cobre. Um estudo realizado com *Aspergillus fumigatus* demonstrou que o bombeamento de cobre no fagolisossomo de macrófagos, por meio de uma ATPase (ATP7A), é uma estratégia utilizada para matar células do fungo, induzindo radicais livres por reação de Fenton-Cobre (WIEMANN et al., 2017).

Tendo em vista a importância do cobre na atividade de importantes enzimas e dos danos que o acumulo deste metal pode causar, os microorganismos necessitam de um

sistema robusto de homeostase deste metal, composto por proteínas que asseguram a adequada acumulação, distribuição e desintoxicação deste metal (KIM; NEVITT; THIELE, 2008; LINDER; HAZEGH-AZAM, 1996). A tabela 1 mostra diferentes classes de proteínaa, já caracterizadas em diferentes organismos, que desempenham funções relacionadas à homeostase do cobre, garantindo níveis adequados do metal no interior do fungo.

Proteínas que compõem a família de proteínas transportadoras de cobre (Ctr), possuem três domínios transmembrânicos. A região amino-terminal, rica em metionina, é voltada para porção extracelular. A porção carboxi-terminal é voltada para a parte intracelular e contém regiões conservadas ricas em cisteína e histidina (JIANG et al., 2005; PUSHIE et al., 2015). Ctr1p e Ctr3p são proteínas de membrana, transportadoras de cobre de alta afinidade, descritas em *S. cerevisiae*, que desempenham importante papel no tráfico do cobre do meio extra para o meio intracelular (PEÑA; PUIG; THIELE, 2000; PUIG et al., 2002). A proteína Ctr4p desempenha papel importante na homeostase do cobre e na virulência de *Cryptococcus neoformans*. Proteína Ctr2p, transportadora de cobre de baixa afinidade, presente no vacúolo, desempenha um papel duplo no processo de homeostase do cobre. Em momentos de aumento na disponibilidade do metal, esta proteína promove o armazenamento no vacúolo celular. Quando há a privação do metal, esta proteína promove a liberação do cobre armazenado, restabelecendo assim níveis normais deste metal (PUIG et al., 2002; SILVA et al., 2011).

**Tabela 1 –** Proteínas de homeostase de cobre caracterizadas em *Saccharomyces cerevisiae, Candida albicans* e *Cryptococcus neoformans* e seus homólogos em *P. brasiliensis.* Adaptado de: (SILVA et al,. 2011).

| Organismo | | | | Descrição |
|---|---|---|---|---|
| *S. cerevisiae* | *C. albicans* | *C. neoformans* | *P. brasiliensis* | |
| **Fatores de transcrição** | | | | |
| Ace1p (Cup2p) | Cup2p | - | - | Excesso de cobre |
| Mac1p (Cua1p) | Mac1p | - | - | Privação de cobre |
| - | - | Cuf1p | Cuf1p | Dupla função, privação e excesso |
| **Transportadores de cobre** | | | | |
| Ctr1p | Ctr1p | Ctr1p | - | Transportador de cobre de alta afinidade |
| Ctr3p | Ctr4p | Ctr4p | Ctr3p | Transportador de cobre de alta afinidade |
| Ctr2p | Ctr2p | Ctr2p | Ctr2p | Transportador de cobre de baixa afinidade |
| Ccc2p | Ccc2p | Ccc2p | Ccc2p | ATPase de cobre intracelular |
| - | Crp1p | - | - | ATPase de exportação de cobre |
| **Metalotioneína de cobre** | | | | |
| Cup1p | Cup1p | Cmt1p | - | Metalotioneínas de cobre |
| Crs5p | Crd2p | Cmt2p | - | |
| **Superóxido dismutases** | | | | |
| Sod1p | Sod1p | Sod1p | Sod1p | SOD cu/zn citoplasmático |
| **Chaperonas** | | | | |
| Atx1p | Atx1p | Atx1p | Atx1p | Chaperona para Ccc2 |
| CCSp | CCSp | CCSp | CCSp | Chaperona para SOD |
| **Redutases** | | | | |
| Fre1p, Fre2p, Fre3p, Fre5p, Fre7p e Fre8p | Fre10p | - | Fre3p, Fre7p | Metaloredutase |
| - | Cfl4p | - | - | Metaloredutase |

O sistema de homeostase do cobre é regulado por fatores de transcrição metais responsivos (PEÑA; PUIG; THIELE, 2000; PUIG et al., 2002; SILVA et al., 2011). Em *S. cerevisiae*, fatores de transcrição como Ace1p e Mac1p, atuam de forma independente

na regulação expressão de proteínas transportadoras de cobre. Em momentos de privação do cobre, o fator Mac1p ativa a expressão de proteínas como Ctr1p e Ctr3p. Em situações de aumento dos níveis de cobre, Ace1p ativa a expressão de metaloproteínas e da Sod1p, como forma de proteção contra a toxicidade deste íon (KELLER; BIRD; WINGE, 2005). O fator de transcrição Cuf1p, que regula a expressão da proteína Ctr4p, desempenha papel importante no crescimento e na virulência do fungo *Cryptococcus neoformans* em infecções no tecido cerebral, onde a privação, ou mesmo o excesso de cobre, são utilizados como estratégia de defesa pelas células deste tecido (WATERMAN et al., 2007). Após a passagem do cobre do meio extra para o meio intracelular, o metal se liga a algumas chaperonas que transportam este metal para enzimas citosólicas cobre dependentes e organelas, como a mitocôndria (JIANG et al., 2005; PUSHIE et al., 2015; SILVA et al., 2011).

A enzima ATPase tipo-p, Ccc2p, presente na face trans do complexo de Golgi, recebe o cobre por meio da chaperona ATX1 (PUIG et al., 2002b). O cobre atua como importante cofator, sendo essencial em enzimas como a citocromo c oxidase (DING et al., 2014). A chaperona Cox17p, localizada no citosol e no espaço intermembrana da mitocôndria, é um doador de cobre para a Sco1p, uma proteína importante na transferência de cobre para citocromo c oxidase (GLERUM; SHTANKO; TZAGOLOFF, 1996). A chaperona de cobre para superóxido dismutase (CCS) desempenha um papel importante como transportadora do $Cu^{1+}$ para a enzima superóxido dismutase cobre/zinco dependente (Sod1p). Um estudo recente demonstrou que um domínio N-terminal (Domínio 1) nesta chaperona é o responsável pela transferência de cobre para Sod1p, ativando esta enzima (FUKUOKA et al., 2017).

A enzima Sod1p possui atividade antioxidante e, juntamente com outras enzimas, como as catalases e peroxidases, minimiza o dano oxidativo, favorecendo assim a sobrevivência do fungo. O cobre presente no centro ativo desta enzima é reduzido pelo superóxido de oxigênio ($^{\bullet}O_2$), dando origem ao oxigênio molecular e ao peróxido de hidrogênio (BEYER; IMLAY; FRIDOVICH, 1991; GRALLA; VALENTINE, 1991). A atividade antioxidante da enzima Sod1p e sua relação com a virulência do patógeno foi demonstrada em um estudo no qual se avaliou a susceptibilidade de mutantes de *C. neoformans* que tiveram o gene que codifica esta proteína silenciado. (COX et al., 2003). Estudos proteômicos que analisam o perfil de expressão de proteínas de fungos, na presença de estresse oxidativo, têm reforçado a importância desta enzima para

sobrevivência destes microorganismos (LIMA et al., 2014; PARENTE-ROCHA et al., 2015).

O cobre no estado oxidado é incapaz de passar pela membrana plasmática fungica, tornando necessária a presença de um sistema de captação eficiente, que facilite a importação pela camada bilipídica da membrana. Neste processo, a redução do cobre de $Cu^{2+}$ para $Cu^{1+}$ é necessária (HASSETT; KOSMAN, 1995). O cobre é reduzido por metaloredutases como Fre1p e Fre2p, sendo esta redutase depentende do fator do fator de transcrição Mac1p. A redução do cobre, de seu estado cúprico ($Cu^{2+}$) para cuproso ($Cu^{1+}$), facilita não apenas a entrada do cobre do meio extracelular para o meio intracelular, como também para o interior de organelas como mitocôndria (GEORGATSOU et al., 1997; JUNGMANN et al., 1993; SMITH; LOGEMAN; THIELE, 2017). Metalotioneínas desempenham papel importante na homeostase de cobre reduzindo níveis deste metal no citoplasma. Em *S. cerevisiae*, duas metalotioneínas, Cup1p e Crs5p, são ativadas pelo fator de transcrição Ace1p (THIELE, 1988). A figura 6 traz uma representação do sistema de homeostase do cobre em em *S. cerevisiae* e *C. neoformans*, nos quais se observam as principais proteínas já caracterizadas neste processo.

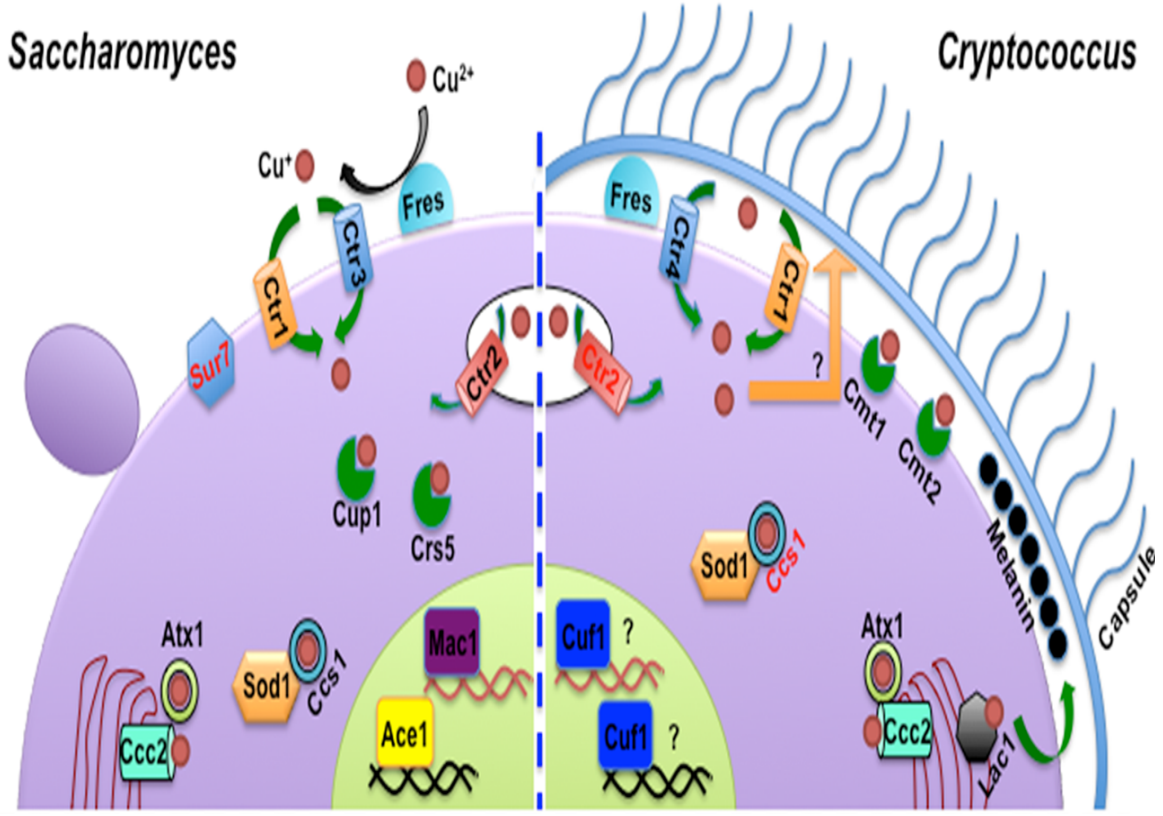


**Figura 6** – Representação do sistema de homeostase do cobre em *S. cerevisiae* e *C. neoformans*. Ctr1p, Ctr3p e Ctr4p (captadores de cobre de alta afinidade), Ctr2 (captador de cobre de baixa afinidade), Fresp (ferro redutases), Sod1p (superóxido dismutase cobre/zinco dependente), Ccs2p (chaperona de cobre para Sod), Cup1p, Crs5p, Cmt1p e

Cmt2p (metalotioneínas), Atx1p (Chaperona de cobre), Ccc2p (Atpase-tipoP), Mac1p, Ace1p, Cuf1p (fatores de transcrição cobre dependente). Adaptado de: (DING et al., 2014).

Dentre os processos celulares nos quais a presença do cobre é necessária, o processo de obtenção de energia pela via aeróbica e o de detoxificação merecem destaque (Figura 7). No processo de respiração aeróbica a citocromo c oxidase é uma importante enzima, promovendo a transferência de elétrons para o oxigênio. Este evento ocorre no interior da mitocôndria, durante o processo da fosforilação oxidativa. Esta enzima tem o cobre como um importante cofator no processo de redução do oxigênio. Já na detoxificação, a superóxido dismutase depentente de cobre/zinco, encontrada no citoplasma, é fundamental para que o microorganismo minimize a ação de radicais livres (BESOLD; CULBERTSON; CULOTTA, 2016; COX et al., 2003; HORNG et al., 2004; SMITH; LOGEMAN; THIELE, 2017).

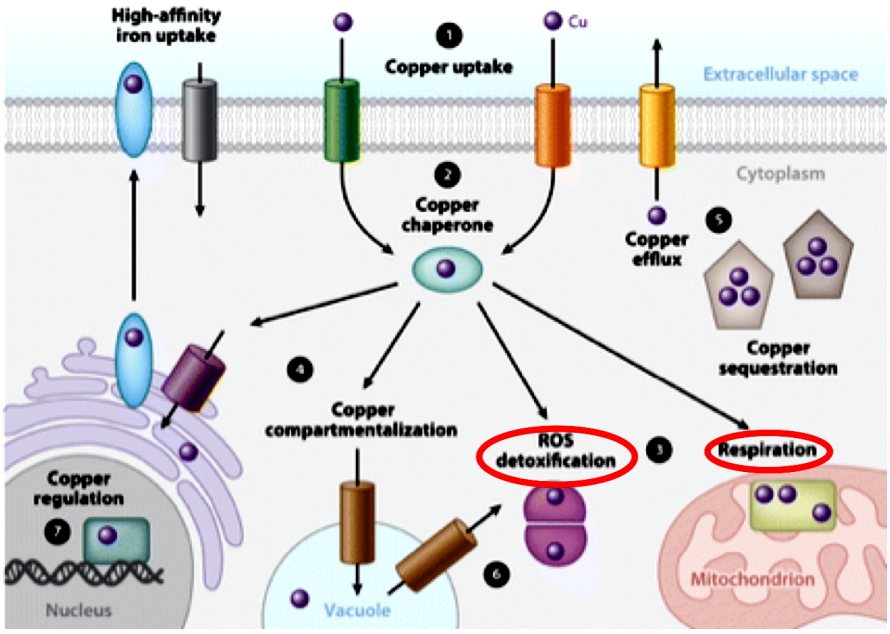


**Figura 7 –** Resumo do processo de captação e distribuição do cobre em células fúngicas. (SMITH; LOGEMAN; THIELE, 2017)

## 2. Justificativa

Fungos do gênero *Paracoccidioides brasiliensis* são os agentes etiológicos da principal micose sistêmica em países da América Latina, sendo Brasil uma área endêmica desta micose. Nesse sentido, o estudo e caracterização das respostas de um representante deste complexo de fungos patogênicos, poderá elucidar em partes os mecanismos adaptativos empregados por estes patógenos para sua sobrevivência durante a privação de cobre imposta pelo hospedeiro.

Durante o estabelecimento da infecção vários mecanismos são empregados pelo patógeno e hospedeiro, com o intuito de promover ou inibir o desenvolvimento da doença. Uma das estratégias utilizadas pelo hospedeiro, com intuito de inibir a sobrevivência do patógeno, é denominada imunidade nutricional. Essa imunidade é caracterizada pela limitação de micronutrientes ao patógeno nos fluidos corporais ou no interior de células do hospedeiro. O cobre é um dos metais que é restrito ao patógeno durante o mecanismo de imunidade nutricional. Esse elemento químico desempenha um papel importante em processos celulares essenciais para sobrevivência e virulência de vários microrganismos.

Em fungos do gênero *Paracoccidioides*, a limitação de micronutrientes como ferro e zinco afeta diretamente o estabelecimento da micose, entretanto, embora o cobre atue como cofator de várias enzimas, poucos estudos abordam a influência desse metal no metabolismo de *Paracoccidioides spp*.. Tais fungos, são os agentes etiológicos da principal micose sistêmica em países da América Latina, sendo Brasil uma área endêmica desta micose. Portanto, o estudo e caracterização das respostas de um representante deste complexo de fungos patogênicos, poderá elucidar em partes os mecanismos adaptativos empregados por estes patógenos para sua sobrevivência durante a privação de cobre imposta pelo hospedeiro. Neste sentido, análise proteômica, utilizando a tecnologia iTRAQ, fornece ao pesquisador uma leitura mais precisa na quantificação de proteínas e, consequentemente, das respostas metabólicas empregadas pelo patógeno para o estabelecimento da infecção.

## 3. Objetivo geral:

Realizar uma análise proteômica de *Paracoccidioides brasiliensis* (*Pb*18), cultivado sob privação de cobre e identificar as alterações metabólicas deste fungo durante esta condição.

### 3.1. Objetivos específicos:

- Avaliar o nível de expressão relativa do transportador de cobre de alta afinidade Ctr3, em condições de privação deste metal;
- Determinar tempo de cultivo, sob condições de privação deste metal para extração de proteínas e análise proteômica;
- Avaliar se *P. brasiliensis* sofre privação de cobre durante infecção em macrófagos;
- Categorizar funcionalmente as proteínas identificadas a partir da análise proteômica;
- Realizar testes bioquímicos e marcação com corantes fluorescentes para validar os dados obtidos através das análises proteômicas;
- Propor possíveis estratégias moleculares de *P. brasiliensis* frente a adaptação durante a privação de cobre.

# Capítulo II

# Metabolic adaptation of *Paracoccidioides brasiliensis* in response to copper deprivation.

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**Abstract**
Copper is an essential micronutrient for the performance of important biochemical processes such as respiration, detoxification and uptake of metals such as iron. Studies have shown that copper deprivation is a strategy used by the host against pathogenic fungi such as *Cryptoccocus neoformans* and *Candida albicans* during growth and development of infection in the lungs and kidneys. Although there are some studies, little is known about the impact of copper deprivation in members of the *Paracoccidioides* genus. Therefore, using isobaric tag labelling (iTRAQ)-Based proteomic approach and LC-MS/MS, we analyzed the impact of copper deprivation in the metabolism of *Paracoccidioides brasiliensis*. One hundred and eight three (183) differentially abundant proteins were identified when yeast cells were deprived of copper, which affected cellular respiration and detoxification processes. Changes in cellular metabolism such as increased beta oxidation and cell wall remodeling were described.

**Keywords: Copper depletion; Pathogenic fungus; Proteomic analysis.**

## INTRODUCTION

Based on the ability to cycle between reduced ($Cu^{1+}$) and oxidized states ($Cu^{2+}$) the metal copper is an essential micronutrient to organisms. Copper acts as a cofactor in enzymes such as superoxide dismutase Cu/Zn (Sod1p), an important detoxification enzyme, and cytochrome c oxidase (Coxp), fundamental to the process of cellular respiration. Copper in the cupric ($Cu^{2+}$) state is reduced to $Cu^{1+}$ by iron reductases (Fre1p, Fre2p) in the extracellular medium [1] and transported into cells by high affinity transporters (Ctr1p, Ctr3p, Ctr4p) [2,3]. Then, copper is distributed by chaperones as Ccs1p, which has an N-terminal domain responsible for transferring the metal to Sod1p, being this process fundamental for the activation of this enzyme [4]. The Atx1p chaperone, delivers copper to a P-type ATPase (Ccc2p) present in the membrane of the Golgi complex [5]. In *Saccharomyces cerevisiae* Atx1p is described as a protein that

protects against reactive oxygen species, when there is a compromise in the activity of Sod1p [6,7]. Furthermore, the Cox17p chaperone is an important copper donor for cytochrome c oxidase (Coxp) [8], present in the respiratory chain complex IV. The enzyme is a terminal oxidase of the respiratory chain and presents a highly conserved heme-copper catalytic structure [9].

Due to the fact that copper is an essential trace element presenting toxic properties when in excess, organisms developed sophisticated mechanisms to provide the micronutrient to biological processes while preventing $Cu^{1+}$ toxicity, which includes the increase in production of toxic hydroxyl radicals, damaging DNA and proteins [10,11]. Mechanisms allowing the homeostasis of copper at an appropriated concentration operates at several levels. Copper homeostasis in fungi is obtained at both transcriptional and post-transcriptional levels [12]. In the model fungus *Saccharomyces cerevisiae* cell surface metaloreductases Fre1p and Fre2p reduces Cu (II) to Cu (I) [13], and the high-affinity transporters Ctr1p and Ctr3p, regulated at transcriptional level by copper availability, are responsible for the metal uptake [14].

In addition, stringent control of Cu uptake is also critical for avoiding excessive intracellular accumulation and toxicity [15]. Under conditions of exposure to a high-Cu environment, *S. cerevisiae* transcriptional factor *Mac1* is rapidly degraded, and that response is accompanied by decreased expression of the target Ctr transporters at both the transcriptional and posttranslational levels [16]. Another transcriptional factor responsive to excessive copper is *Ace1*; under these conditions this transcriptional factor induces the expression of copper detoxification genes such as *Sod1*, *Cup1* and *Crs5* [7]. In *Candida albicans* the response to copper excess involves the activation of the copper extrusion pump Crp1p, responsible for transporting copper from the cytoplasm to the extracellular environment and expression of copper metallothionein Cup1p, involved in binding to copper in conditions of high concentration, inside the cells [17].

An important aspect of host-pathogen interaction is the homeostasis of cooper during infection. In this sense, the host can sequester copper in a process known as nutritional immunity preventing the pathogen from obtaining the metal. For example, the opportunistic fungal pathogen *C. albicans* faces copper nutritional immunity since the calprotectin protein from host, sequester Cu by binding Cu(II) with subpicomolar affinity, inducing cooper starvation [18].

Members of the *Paracoccidioides* genus are the causative agents of paracoccidioidomycosis (PCM), a fungal disease highly prevalent in Latin America

[20,21]. The infection begins after inhalation of conidia or mycelial fragments that reach the alveoli where they differentiate into yeast cells, a process highly dependent on temperature and crucial for the disease establishment [22,23]. Several studies have demonstrated the importance of copper for different organisms [12,15,19]. However, little is known about the impact of this metal deprivation in the genus *Paracoccidioides*. Previous *in silico* analysis have shown that *Paracoccidioides* spp. have homologous copper homeostasis-related proteins such as metalloreductases (Fre1p, Fre3p), high and low affinity copper transporters Ctr3p and Ctr2p respectively, copper metalloregulatory transcription factor (*Mac1*), metallochaperone (Atx1p), transporting P-type ATPase (Ccc2p), as well as enzymes related to the detoxification process (Sod1p, Sod2p)[24].

In this work we demonstrated that *Paracoccidioides brasiliensis*, faces copper deprivation upon macrophage infection. Besides, we observed that copper depletion affected the activity of cytochrome c oxidase a process accompanied by increase in the expression of the enzyme alternative oxidase (Aoxp), associated with alternative respiratory pathway. A decrease in the expression of Sod1p, copper dependent, affected the detoxification capacity, leading to increased expression of thioredoxin and superoxide dismutase Mn/Fe (Sod2p). Enzymes of the pentose phosphate pathway related to the production of NADPH, associated with detoxification processes, as well a chaperone (Atx1p), were increased. Up-regulation of proteins of beta-oxidation, glyoxylate cycle, and remodeling of cell wall were also identified.

## MATERIALS AND METHODS

### Microorganism and growth conditions

*Paracoccidioides brasiliensis Pb*18 (ATCC 32069) was employed in this work. The yeast cells were cultivated for 4 days, at 36°C in BHI solid medium supplemented with 4% (w/v) glucose. For the experiment of copper depletion *P. brasiliensis* yeast cells, were incubated in McVeigh/Morton medium (MMcM) [21]. The copper depletion medium was prepared without $CuSO_4$ and supplemented with 50 μM bathocuproinedisulfonic acid (BCS). For control, the medium was supplemented with 10 μM $CuSO_4$.

### *Paracoccidioides brasiliensis* cell viability analysis

After cultivation of yeast cells, viability was determined by membrane integrity analysis using propidium iodide as dead cells marker, as previously described [25]. Yeast

cells (5 x $10^6$ yeast cells/mL) were centrifuged at 3500 *g* at 4ºC for 5 min and the supernatant was discarded. Propidium iodide (1μg/mL) was added to the cell's suspension for 20 min in the dark, at room temperature and cells were analyzed by flow cytometer (BD® Accuri C6 Flow Cytometer). A minimal of 10,000 events per sample was acquired with the FL-3-H channel.

**Macrophage infection and generation of macrophage ATP7a-silenced cells.**

Murine macrophage cell line J774 A. 1 (BCRJ Cell Bank, Rio de Janeiro, accession number 0121) was employed. The macrophages were maintained in RPMI medium (RPMI 1640, Vitrocell, Brazil) supplemented with non-essential amino acids (Sigma-Aldrich, St. Louis, MO), 10% (w/v) and fetal bovine serum (FBS), at 37°C in 5% $CO_2$. 1x$10^6$ macrophages were seeded into each well of a 24 well tissue plate and IFN-γ (1 U/mL) (Sigma–Aldrich, St. Louis, MO, United States) was added for 24 h at 37°C in 5% $CO_2$ for macrophage activation,

ATP7a silenced macrophage cells was generated by transient transfection of a siRNA double-stranded, against ATP7a gene (Silencer siRNA mouse ATP7a Cat. No. AM16708, ThermoFisher Scientific, Waltham, MA), using Lipofectamine 2000 as transfection reagent, following the Lipofectamine 2000 protocol (Invitrogen, Cat. No. 11668-027), in RMPI 1640 medium without any supplementation. A scramble siRNA was used as transfection negative control (Silencer Negative Control Cat. No. AM4611, ThermoFisher Scientific, Waltham, MA).

After 24, 48 and 96 h macrophages viability were confirmed by microscopy using Trypan blue dye. Trizol was added in each well and total RNA was isolated. RNAs from non-silenced macrophages were obtained as control. After reverse transcription, the ATP7a inhibition was evaluated by qRT-PCR, using TaqMan gene expression assay (ThermoFisher Scientific, Waltham, MA; ATP7a TaqMan Cat. No. 437663). The transcript of α-tubulin (TaqMan Cat. No. 492936) was used for normalization of transcript amplification.

For fungal burden analysis in ATP7a silenced macrophages, 5x$10^6$ cells/mL of silenced and non-silenced macrophages were co-cultivated with $10^6$ yeast cells/mL in RMPI 1640 medium without supplementation. The cells were co-cultivated for 24 h at 37°C in 5% $CO_2$ to allow fungal internalization. Each well was washed twice with 1 ml of PBS 1x in order to get rid of non-internalized yeast cells. Infected macrophages were lysed with ice-cold ultrapure sterile water, and dilutions of the lysates containing the

phagocytized yeast cells were plated in BHI agar and incubated at 37°C in 5% $CO_2$ atmosphere. After 7 days of incubation the number of CFU was determined.

**Protein extraction**

Yeast cells cultivated in presence and absence of copper were harvested at 24 h time-point and centrifuged at 5000 *g*, 4ºC for 10 min. The supernatant was discarded, and the cells washed three times with PBS 1X. Posteriorly, the cells were resuspended in extraction buffer containing 20 mM Tris–HCl pH 8.8; 2 mM $CaCl_2$ with a mixture of nuclease and protease inhibitors (GE Healthcare). After this step, the cells were distributed in tubes, glass beads were added, and the cells were disrupted on ice in a bead beater apparatus for 5 cycles of 30 s. Next, the cells were centrifuged at 10,000 *g* for 10 min at 4ºC, three times and the quantification of protein extracts was performed as described [ 26].

**In-solution Protein Digestion**

150 µg of protein extract of each condition/replicate was prepared for trypsin digestion [27]. To the protein suspension was added ice-cold acetone, followed by incubation overnight at -20°C. After, the samples were centrifuged at 20,000 x *g* for 15 min at 4°C and re-suspended in 8 M urea in 0.05 M triethylammonium bicarbonate buffer (TEAB), pH 7.9. Next, proteins were reduced with 0.005 M DTT for 25 min at 55°C and alkylated with 0.014 M iodoacetamide for 40 min at room temperature, in dark. Samples were diluted 5-fold with 0.001 M $CaCl_2$ in 0.025 M TEAB, pH 7.9. Modified trypsin (Promega, Madison, WI, USA) was added in 1:50 (w/w) substrate ratio, and samples were incubated overnight at 37°C, followed by acidification with 0.1% (v/v) TFA and desalting on homemade C18 microcolumns in P200 low-binding tips. Thereafter, all samples were lyophilized in speed vacuum [27].

**Isobaric Tag Labeling**

Biological triplicates of protein samples from yeast cells were labeled with iTRAQ as previously described [28]. Desalting of the samples was performed. Then, 50 µg of the material was resuspended in 17 µL of 300 mM TEAB. The iTRAQ marker (Reagents Multiplex Kit, Sigma Aldrich) resuspended in 70 µl of ethanol, was then added. The samples were incubated for 2 h at room temperature. Equimolar amounts of iTRAQ label were mixed in all samples (copper starvation labeled with 114; control; with 115).

**LC-MS/MS**

iTRAQ labelled peptides were analyzed in three technical repetitions each and fractionated using a nano-UHPLC Dionex Ultimate 3000 (Thermo Fisher Scientific) coupled with an Orbitrap EliteTM Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) as previously described [27]. Each fraction was loaded onto a pre-column (110 µm x 200 nm) packed in-house with C18 ResiproSilPur of 5 µm with 120 Å pores (Dr. Maisch GmbH, Ammerbuch, Germany). Second chromatography was carried out in column (75 µm x 35 nm) packed in- house with C18 ResiproSilPur of 3 µm with 120 Å pores (Dr. Maisch GmbH, Ammerbuch, Germany) and eluted using a gradient from 100% solvent A [0.1% (v/v) formic acid] and 26% solvent B [0.1% (v/v) formic acid, 95% (v/v) acetonitrile] for 180 min, followed from 26% to 100% solvent B for 5 min and 100% solvent B for 8 min (a total of 193 min at 200 nL/min). After each run, the column was washed with 90% solvent B and re-equilibrated with solvent A. Mass spectra were acquired in positive ion mode applying data-dependent automatic survey MS scan and tandem mass spectra (MS/MS) acquisition modes. Each MS scan in the Orbitrap analyzer (mass range = $m/z$ 350–1800, resolution = 120,000) was followed by MS/MS of the fifteen most intense ions in the LTQ. Fragmentation in the LTQ was performed by high-energy collision-induced dissociation (HCD), and selected sequenced ions sequences were dynamically excluded every 15 s.

**MS/MS spectra processing**

Raw data processing used Proteome Discoverer v.1.3 beta (Thermo Scientific). Searches of the Raw files used Proteome Discoverer with Mascot v.2.3 algorithm against the *P. brasiliensis* database, downloaded using the Database on Demand tool in UniProt/SWISS-PROT (http://www.uniprot.org/) and NCBI (www.ncbi.nlm.nih.gov/genome/?term=Paracoccidioides) database. For false discovery rates, the number of proteins, protein groups and peptides were filtered to values below 1%. Using Proteome Discoverer two peptides per protein was the minimum accepted value for identification.

**Data Analysis**

The data consisted of three replicates containing global proteome. To increase the reliability, the acceptance criteria were applied, as following: proteins identified with at

least 1 single peptide; with high or medium FDR and in at least 2 of 3 replicates. For statistical analysis, it was applied unpaired Student's *t*-test. Statistical difference was set at p-value= ≤ 0.05. Functional categories were determined by search in Blast2GO platform (http://www.blast2go.com/b2ghome), Pedant on MIPS-Functional Catalogue (http://pedant.helmholtz-muenchen.de/) and KEGG database (http://www.genome.jp/kegg/). Sequence annotation was assessed using a BlastP algorithm (http://blast.ncbi.nlm.-nih.gov/Blast.cgi).

**cDNA synthesis and quantitative real time PCR (RT-qPCR) analysis**

Yeast cells were harvested at time-points 0, 4, 8, 12 and 24 h in copper deprivation. Then, cells were treated with TRIzol (SIGMA-ALDRICH) and the RNA was extracted following the manufacture's protocol. Then, total RNA was treated with DNase (RQ1 RNase-free DNase, Promega) and subjected to reverse transcription (SuperScript III First-Strand Synthesis SuperMix; Invitrogen, Life Technologies) following the manufacturer's recommendation. SYBR green PCR master mix (Applied Biosystems, Foster City, CA) was used in the RT-qPCR assays performed in a Step OnePlus system (Applied Biosystems). Normalization used the gene encoding the L34 protein (PAGD_04085). Standard curves were generated by 1:5 dilution of the cDNA, and the relative expression levels of the transcripts were calculated using the standard curve method for relative quantification [29]. The used oligonucleotides are described in (**Supplementary Table S1**).

**Analysis of chitin and glucan amount in the cell wall of *Paracoccidioides brasiliensis* in copper deprivation**

Calcoflour White (CFW, Sigma-Aldrich) and Aniline Blue (AB, Sigma-Aldrich) were used to stain *P. brasiliensis* (*Pb*18) yeast cell in order to evaluate the effect of copper depletion in the composition of cell wall, since CFW and AB specifically binds chitin and glucan respectively. *P. brasiliensis* grew in copper depletion or in presence of this metal for 24 h. The cells were subjected to staining with the dyes described above and analyzed by fluorescence microscopy as already detailed by de Curcio and colleagues 2017 [30].

**Analysis of mitochondrial activity of *P. brasiliensis* in copper deprivation**

The mitochondrial activity was monitored using the Rhodamine 123 (Sigma-Aldrich) and Mitotracker Green FM (Sigma-Aldrich) fluorescent dye. Yeast cells were stained

according to the manufacturer's instructions. Initially, yeast cells were harvested by centrifugation of 8000 *g* for 10 min at 4°C, diluted in 1× PBS to $10^6$ cells/mL and stained during 45 min at 37ºC with Mitotracker (400nM), Then, the cells were washed with PBS and labeled with Rhodamine (2.4 µM) for 45 min at 37ºC. Afterwards, the cells were washed with PBS and analyzed by fluorescence microscopy (Zeiss Axiocam MRc – Scope A1). The calculation of fluorescence intensity was performed [31]

**Reactive oxygen species evaluation**

The generation of reactive oxygen species (ROS) was evaluated using dichlorofluorescein 2', 7'-diacetate (DCFDA) (Sigma-Aldrich). *P. brasiliensis* yeast cells were cultivated in copper deprivation and collected at 12 h and 24 h of treatment. The cells were harvested by centrifugation of 2000 *g* for 5 min at 4°C, diluted in PBS 1× to $10^6$ cells/mL. Then, 1 mL was transferred to a clean eppendorf, added of 1 µL of DCFDA and the mixture was allowed to stand for 30 min, in dark environment. The cells were washed twice with PBS 1× and observed under fluorescence microscopy (Zeiss Axiocam MRc – Scope A1) using the 490-516 nm filter (FS09).

**Enzymatic activities and biochemical tests**

For confirmation of differential regulation of some proteins, enzymatic activities and biochemical tests were performed. Cytochrome oxidase (CCO) activity was performed employing the Cytochrome C Oxidase Assay Kit (CYTOCOX1-Sigma Aldrich) according to manufacturer's instructions. The CCO activity was evaluated by a colorimetric assay based on observation of the decrease in absorbance of ferrocytochrome C at 550 nm caused by its oxidation to ferricytochrome C by CCO. Triplicates were obtained for each condition. The results were considered statistically significant at p-values $\leq 0.01$ by Student's *t*-test.

Free thiol levels were determined using the Ellman's reagent, 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB - Sigma Aldrich). A total of $10^6$ yeast cells/mL incubated in the presence or absence of copper, were centrifuged for 5 min at 8000 *g* and lysed by the addition of 0.5 mL lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 50 mM ethylenediaminetetraacetic acid [EDTA], pH 7.2) and glass beads in equal volume of the cellular pellet. After centrifugation and lysis, 100 µL of the supernatant was added to 100 µL of 500 mM phosphate buffer, pH 7.5, followed by the addition of 20 mL of 1 mM DTNB. Absorbance was measured at 412 nm using a plate reader. Triplicates were

obtained for each condition, as previously described [26]. Results were considered statistically significant at p values ≤ 0.01 by Student's t-test.

**Obtaining the recombinant protein Atxp and polyclonal antibodies production**

The vector pET32a::Pb18Atx1p was synthesized by GenOne (Rio de Janeiro, Brazil). Briefly, the *Atx1* sequence from *P. brasiliensis* (PADG_02352) was codon optimized for *Escherichia coli*, and cloned into *Bam*HI restriction site from vector pET32a (Novagen, Merck-Millipore). For bacterial transformation, 150 ng of DNA from plasmid pET32a::Pb18Atx1p was added to the *E. coli* BL21 competent cell suspension and kept on ice for 1 min. The cells were transferred to an electroporation cuvette and subjected to 1800 Volts electric field to allow entry of the plasmid into the cell. After electroporation, cells were resuspended in 900 µL Luria-Bertani (LB) medium and incubated at 37°C with shaking for 1 h. Aliquots were plated in LB medium containing 100 µg/mL ampicillin for selection of the transformed bacteria. Recombinant protein induction was performed by addition of Isopropyl-β-D thiogalactopyranoside (IPTG) at 0.1 mM. Protein extracts were submitted to 12% SDS-PAGE followed by coomassie blue staining. The bacteria lysis was performed by adding 500 µg/mL lysozyme for 1 h, under constant stirring, at room temperature and sonication in 5 pulses of 10 min. The supernatant from the solubilization process was used for purification of the recombinant Atx1p by nickel column affinity chromatography.

Purified *P. brasiliensis* recombinant Atx1p was inoculated into 6-8-week-old male BALB/c mice to produce polyclonal antibodies. The purified protein was cut from the polyacrylamide gel, macerated in grinder with 2 mL of PBS 1 x, and 200 µL of the solution were applied to the mice. Three immunizations were performed, 15 days apart, intraperitoneally. Fifteen days after the last immunization, the animals were anesthetized with sodium thiopental (40 mg/kg) and lidocaine hydrochloride (2 mg/kg), and blood puncture was performed. After, the animals were euthanized by cervical dislocation. Sera collected from unimmunized mice were used as negative control (preimmune). All procedures were approved by the Animal Use Ethics Committee of the Federal University of Goiás (CEUA-PRPI-UFG), under protocol number 092/17.

**Western blotting and Immunofluorescence assays**

Proteins in SDS-PAGE were transferred to nitrocellulose membrane that was then incubated with polyclonal anti-Atx1p antibodies at 1:2000 dilution, for 2 h at room

temperature. After washing, the membranes were incubated with peroxidase-coupled mouse anti-IgG secondary antibody (1:1000 dilution). The reaction was revealed by chemiluminescence with the ECL Western Blotting substrate kit (GE Healthcare). Negative control was obtained with preimmune mouse serum (1: 500 dilution).

For immunofluorescence**,** $10^6$ yeast cells/mL were fixed in ice cold pure methanol for 3 h at 20°C. After washing with PBS 1 x, part of the fixed cells was permeabilized with 0.25% triton X-100 (Sigma-Aldrich) for 20 min. Subsequently, cells were incubated for 30 min at room temperature, in the dark in blocking buffer containing 3% (w/v) bovine serum albumin (BSA-Sigma), 0.2% (v/v), tween 20 in PBS, followed by incubation with the primary anti-Atx1p polyclonal antibodies at 1:500 dilution, for 1 h. Subsequently it was added fluorescein isothiocyanate-labeled mouse secondary antibody - FITC (Sigma) at 1:750 dilution, for 1 h. Cells were washed three times with PBS 1 x. Images were taken in bright field and at 450-490 nm for visualization of FITC fluorophore, using the Axio Scope A1 fluorescence microscope. Digital images were acquired using AxionVision software (Carl Zeiss AG, Germany).

## RESULTS

### *Paracoccidioides brasiliensis* faces copper deprivation inside macrophages

Initially, to understand the influence of copper on the survival of *P. brasiliensis* inside J774A.1 macrophages, we knocked down the P-type copper ATPase pump (ATP7A) of this defense cell. This protein is responsible for translocation of copper from the Golgi for phagolysossome during infection [32]. Data analyzes demonstrated that in ATP7A-silenced macrophages, the number of recovered CFU from *P. brasiliensis* was significantly reduced when compared to control macrophages (**Figure 1A**). In this way, the silencing of ATPase pump (ATP7A) reduces the pumping of copper into the phagolysome, intensifying the deprivation of this metal inside this organelle, further damaging the fungus's survival.

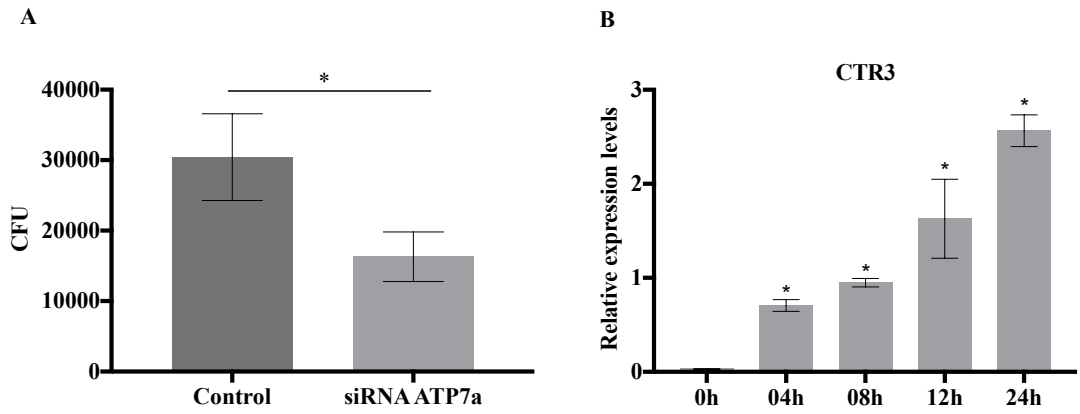


**FIGURE 1.** Recovering of *Paracoccidioides brasiliensis* yeast cells after infection in silenced ATP7A macrophages and fungal response to copper deprivation. **(A)** Colony forming units of *P. brasiliensis* (*Pb*18) cells after 24 h of infection in: Control – non-silenced macrophages, SiRNA ATP7a – ATP7a silenced macrophages. **(B)** Expression analysis of the *Ctr3* gene in yeast cells during copper deprivation: Relative levels of expression of the *Ctr3* gene (Genbank PADG_05084) after treatment with 50 μM BCS at different time-points. Gene expression values were normalized using the expression values of the transcript coding for L34 protein (GenBank PAGD_04085). *Statistically significant ($p < 0.05$).

## Induction of the copper transporter Ctr3-p in response to copper deprivation and fungal viability

The expression profile of the gene encoding the high affinity copper transport Ctr3p in *P. brasiliensis* was evaluated at different times of copper deprivation. After 24 h of deprivation high induction of *Ctr3* was observed (**Figure 1B),** indicating that the fungus lies in copper deprivation. To determine the impact of copper deprivation on fungal viability, flow cytometry was employed. The results demonstrated that, although the fungus was deprived of copper, cellular viability was not affected within the first 24 h **(Supplementary Figure S1)**. After these experiments, a 24 h period for protein extraction and proteomic analysis was employed.

## Proteomic analysis of yeast cells submitted to copper deprivation

A total of 183 proteins were differentially expressed considering $p \leq 0.05$ and were categorized according to their cellular functions using the Functional Catalog (FunCat2). The main biological processes regulated by copper deprivation include metabolism (22%), energy (15%) and protein fate (14%) (**Supplementary Figure S2**). Among the differentially expressed proteins, 173 were up-regulated (**Supplementary Table S2**) and 10 were down-regulated (**Supplementary Table S3**).

## Beta oxidation and glyoxylate cycle increase in yeast cells in copper deprivation

Proteins related to the following pathways, as following: beta-oxidation: acetyl-CoA acyltransferase (PADG_00382 and PADG_01687), acyl-CoA dehydrogenase (PADG_02852), carnitine O-acetyltransferase (PADG_07023); to the glyoxylate cycle: isocitrate lyase (PADG_04709), aconitate hydratase (PADG_11845) malate dehydrogenase (PADG_08059) and to the TCA cycle: isocitrate dehydrogenase (PADG_04249), were increased, strongly connecting beta oxidation and the glyoxylate cycle in yeast cells in copper deprivation (**Figure 2**).

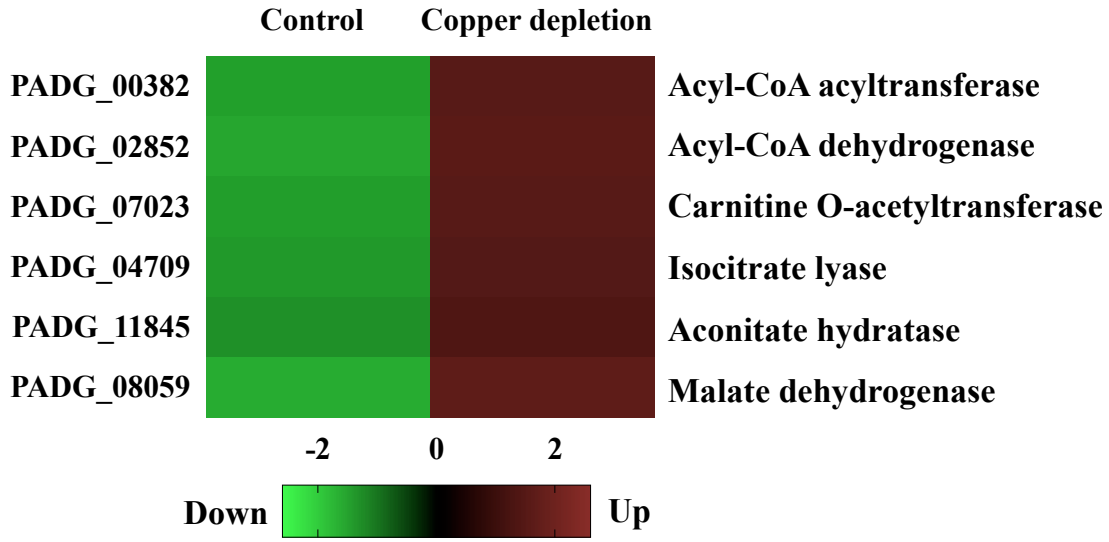


**FIGURE 2.** Beta oxidation-related and glyoxylate cycle proteins up regulated during copper deprivation**.** Heat map showing a group of increased proteins of beta oxidation, TCA and glyoxylate cycle in *P. brasiliensis* yeast cells in copper deprivation.

**Oxidative stress response increase in yeast cells in copper deprivation due to accumulation of ROS**

An important enzyme related to detoxification of *P. brasiliensis*, Zn/Cu superoxide dismutase (Sod1p) (PAGD_07418) was decreased. On the other hand, were induced thioredoxin (PADG_05504), Mn/Fe superoxide dismutase (Sod2p) (PADG_01954), glutathione-S-transferase (GST) (PADG_02526), suggesting that copper deprivation promoted increase in the formation of reactive radicals. Further, we identified increased levels of 6-phosphogluconate dehydrogenase (PADG_03651), enzyme of the phosphate pentose pathway (**Figure 3A**), which may be related to the production of NADPH, an

important reducing agent that increases the efficiency of detoxification enzymes. Decrease of Sod1p accumulation, as well as increase of detoxification enzymes such as thioredoxin, GSTp and Sod2p suggest that copper deprivation has affected the control of free radicals in the cell. Thioredoxin plays an important role in the detoxification process and is related to the regulation of Sod1p activity present in the mitochondrial intermembrane space when there is an increase of free radicals [33]. Evaluation of thioredoxin provided increased enzyme activity during copper deprivation (**Figure 3B**).

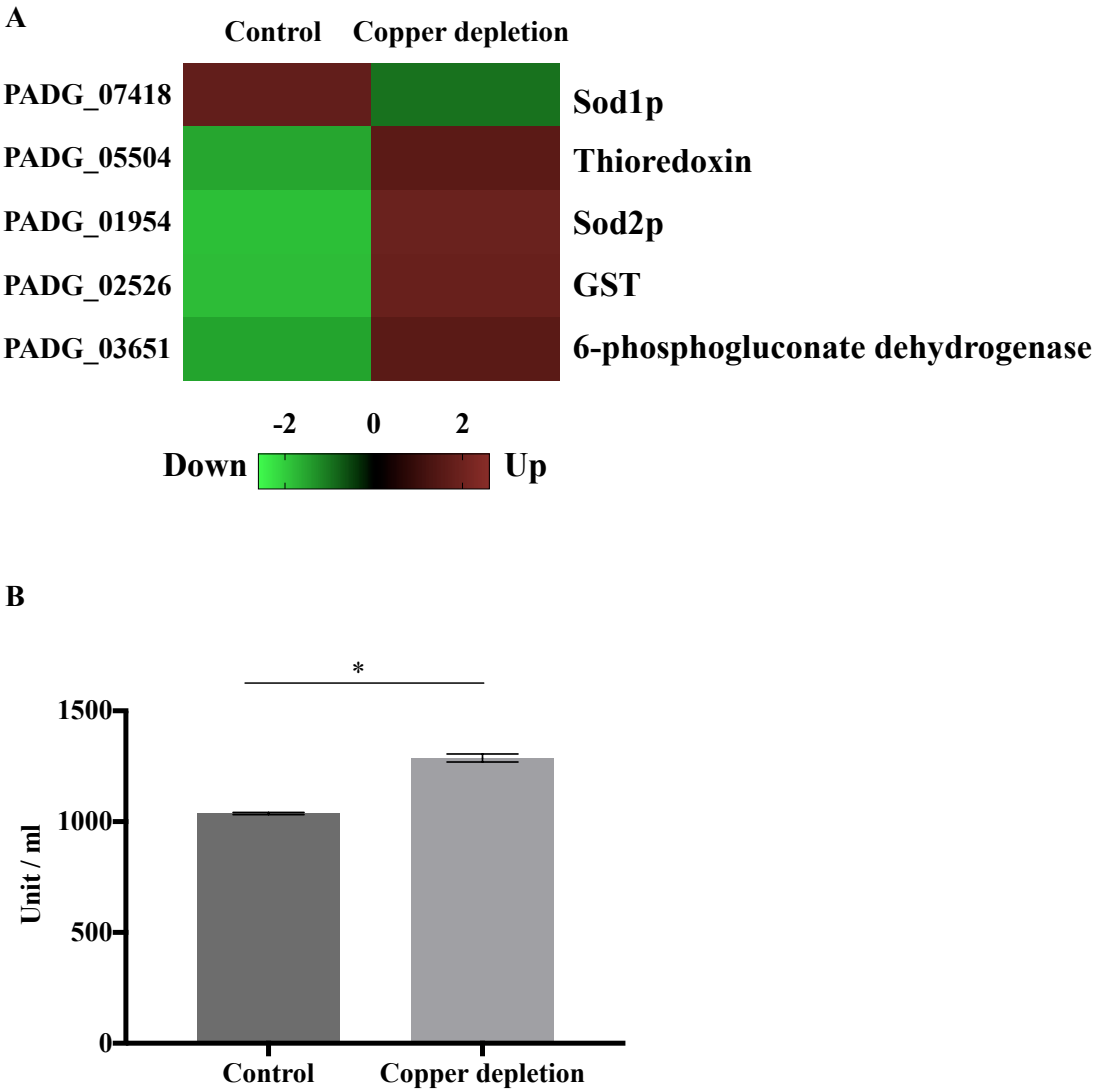


**FIGURE 3.** *Paracoccidioides brasiliensis* responds to oxidative stress during copper deprivation. (**A**) Heat map of proteins related to the response to ROS in *P. brasiliensis* yeast cells. (**B**) The enzymatic activity of thioredoxin in yeast cells treated with BCS for 24 hours. *Statistically significant ($p < 0.05$).

In addition, ROS detection by fluorescence microscopy using 2,7 dichlorofluorescein marker showed increase of ROS after 12 and 24 h in copper-deprived yeast cells compared to control (**Figure 4**). In this way, these data suggest that copper deprivation

induced oxidative stress in yeast cells of *P. brasiliensis*, accounting for the increase in the cells response.

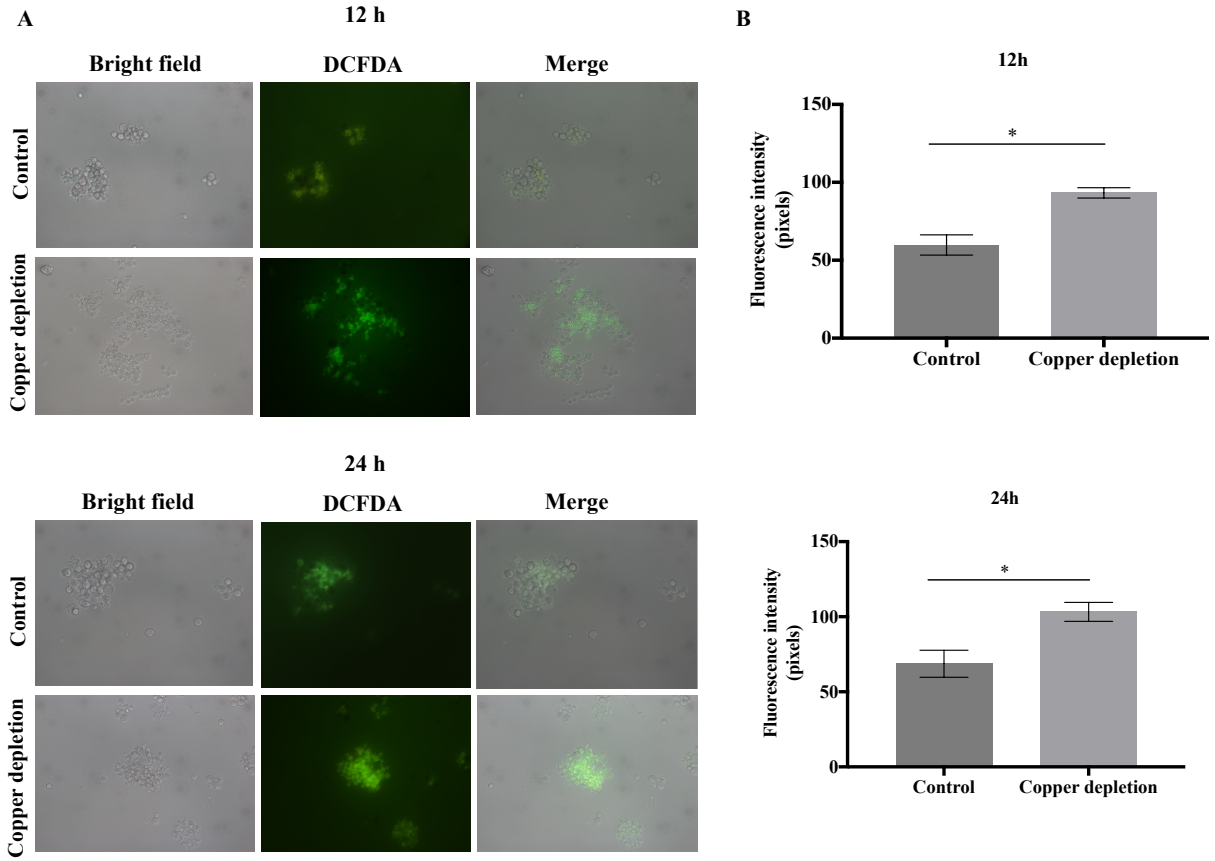


**FIGURE 4.** Copper deprivation results in increase in reactive oxygen species. (**A**) Fluorescence intensity in yeast cells during copper deprivation for 12 h and 24 h. The cells were labelled with dichlorofluorescein 2', 7'-diacetate (DCFDA) and analyzed by fluorescence microscopy. (**B**) The minimum quantity of 50 cells per triplicate were used for the analysis to construct the fluorescence intensity graph, ∗demonstrate a significant difference between the samples with the P-value of ≤0.05. The error bars represent the standard deviation of the samples in triplicates.

## The metabolism of cell wall increases during copper deprivation

Enzymes involved in cell wall synthesis, UDP-N-acetylglucosamine pyrophosphorylase (PADG_04312), UDP-galactopyranose mutase (PAGD_00912) and 1,4-alpha-glucan branching enzyme (PADG_12426), were increased in yeast cells deprived of copper (**Figure 5A**). The images shown in (**Figure 5B**) represent increased fluorescence of yeast cells in copper deprivation when stained with the glucan-binding dye, aniline blue. The fluorescence of chitin was similar in yeast cells deprived or not of copper. In this sense, fluorescence intensity quantification confirms that only the amount of glucan was increased (**Figure 5C**). Possibly glucose produced by gluconeogenesis driven aminoacids could be used in remodeling the fungal cell wall.

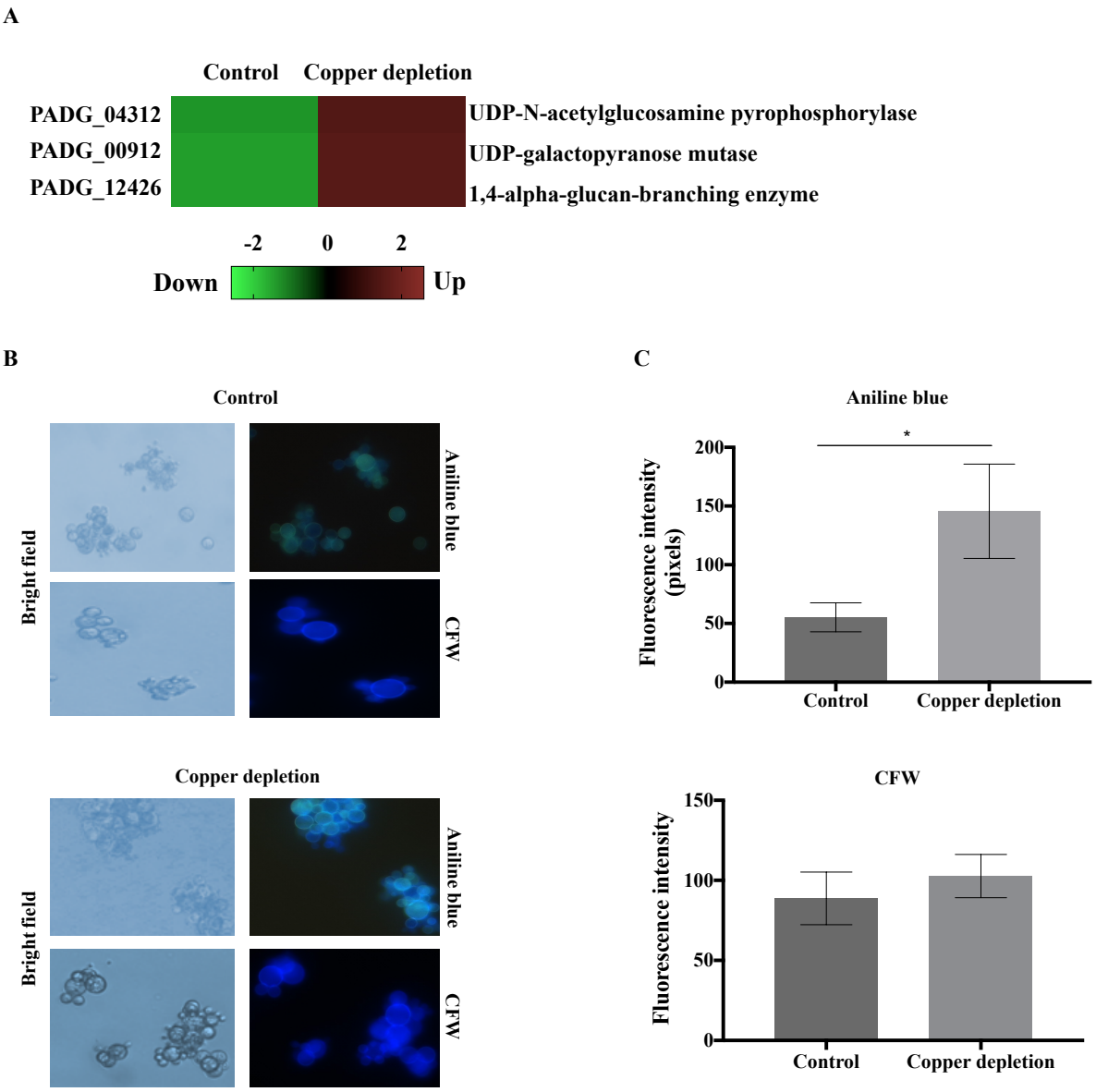


**FIGURE 5.** Evaluation of the cell wall metabolism in yeast cells of *P. brasiliensis* during copper deprivation. (**A**) Heat map showing increased accumulation of proteins related to the cell wall metabolism during copper deprivation. (**B**) Analysis of glucan amount by aniline blue and chitin by Calcofluor White (CFW). (**C**) Fluorescence intensity in control and treatment, both labeled with aniline blue and CFW. To determine significant differences in each condition, pixels intensity values were obtained from triplicates with at least 50 cells, each *indicated P-value of ≤0.05.

## Copper deprivation induces alternative oxidase and decreased mitochondrial activity

The enzyme alternative oxidase (Aoxp) (PADG_03747) increased in yeast cells in copper deprivation (**Supplementary Table S2**). It is described that Aoxp is expressed when there is a compromise in activity of cytochrome c oxidase (Coxp) [34]. Copper is a necessary cofactor for Coxp activity. In this context, the absence of copper could activate the alternative system, mediated by Aoxp, in order to compensate for a possible loss of Coxp activity due to the reduction of copper levels. In fact, we identified alternative

oxidase (PADG_03747) accumulated. Considering the important role of copper in Coxp activity, we hypothesized that the increase in Aoxp accumulation should be related to decreased Coxp activity caused by copper deprivation. In this way, two strategies were performed. The first analysis was to evaluate the enzymatic activity of Coxp. Activity of Coxp was reduced in copper deprivation in comparison to control (**Figure 6A**). Then, we evaluated the activity of the electron transport chain, by fluorescence microscopy. Analysis of rhodamine fluorescence demonstrated mitochondrial electron transport chain repression in copper depletion (**Figure 6B/C**). On the other hand, mitochondrial integrity was not compromised, since mitotracker labeling did not show significant differences between treated and control conditions (**Figure 6B/C**), again indicating that copper deprivation could repress electron transport chain, without affecting the integrity of mitochondria.

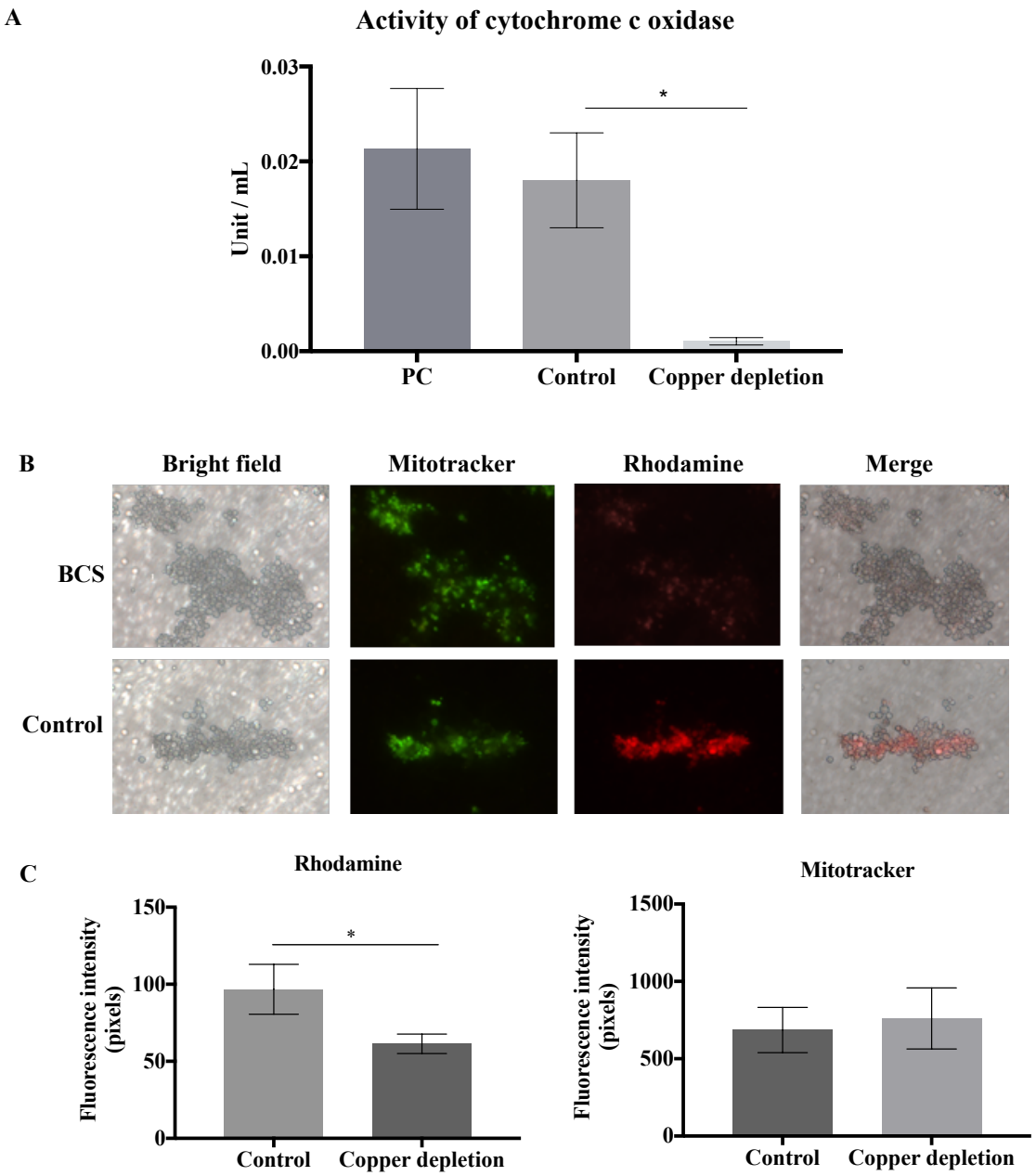


**FIGURE 6.** Activity of cytochrome c oxidase (Coxp) and oxidative phosphorylation are regulated by copper deprivation. (**A**) Yeast cells of *P. brasiliensis* were incubated in the presence and absence of copper, and the enzymatic activity of (Coxp) was evaluated. PC represents de positive control of Assay Kit (Sigma-Aldrich – CYTOCOX1). (**B**) The cells of *P. brasiliensis* were labelled with mitotracker and rhodamine for verification of integrity and mitochondrial activity, respectively. (**C**) Fluorescence intensity, from B. *Statistically significant ($p < 0.05$).

## The cytoplasmic Atx1p copper chaperone is induced in copper deprivation: confirmatory assays

Our proteomics analysis demonstrated that the expression of a copper chaperone named Atx1p (PADG_02352), increased in copper deprivation, as detected by proteomic analysis (**Supplementary Table S2**). Gene expression analysis by qRT-PCR confirmed proteomic data (**Figure 7A**). Using polyclonal anti-Atx1p antibodies,

immunofluorescence of Atx1p in *P. brasiliensis* yeast cells was performed. Fluorescence was observed only when permeabilizing the cell, indicating that Atx1p presents cytoplasmic localization in *P. brasiliensis* (**Figure 7B**).

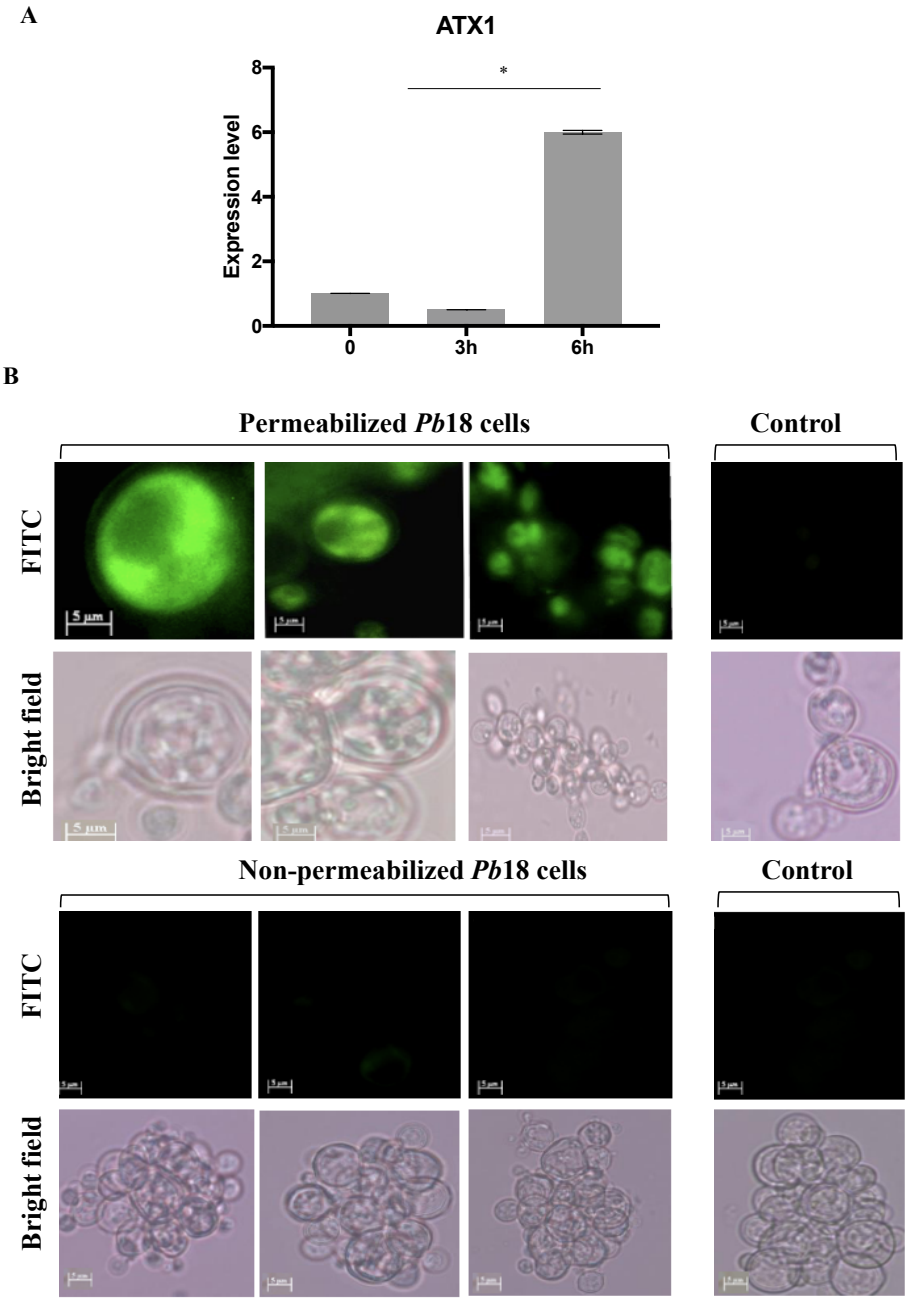


**FIGURE 7.** Expression of transcript and cell localization of Atx1p in *Paracoccidoides brasiliensis.* (**A**) Expression analysis of *Atx1* in yeast cells during copper deprivation. Relative levels of expression of the *Atx1* gene in treatment with 50 mM BCS at different time points. Gene expression values were normalized using the expression values of the transcript coding for tubulin (GenBank accession number (PAGD_04085). *Statistically significant ($p < 0.05$). (**B**) Atx1p immunofluorescence. The immunofluorescence assay was performed with triton X-100 permeabilized and non-permeabilized cells, incubated with anti-Atx1p polyclonal antibodies for 1 h, followed by incubation with the isothiocyanate-labeled mouse anti-IgG secondary antibody fluorescein - FITC for 1 h. Fluorescence was only observed in permeabilized cells. Images were taken in bright field and at 450-490 nm wavelength for viewing FITC fluorophore at 1000X magnification using the Axio Scope A1 fluorescence microscope. Digital images were acquired using AxionVision.

## DISCUSSION

During host infection, pathogenic fungi may find different conditions regarding copper concentration depending on the infected tissue. *C. neoformans* finds high levels of copper in the lungs at the onset of infection and reduced levels of this metal during brain infection [35]. In our study, we infected macrophages silenced for expression of type P copper ATPase (ATP7A) which pumps copper into the phagolysosome, and, after CFU counting we noticed lower fungal cellular recovery, compared to non-silenced macrophages. The copper accumulation inside macrophages seems to be a strategy used to limit the growth of fungi such as *Aspergillus fumigatus* [36]. In this sense, *A. fumigatus* had an increase of the survival during infection in ATP7A-deficient zebrafish [37]. Macrophages silencing for ATP7A reduces the fungicidal activity against *E. coli* [38]. In contrast, the data obtained in this work suggest that *P. brasiliensis* is deprived of copper inside macrophages and that silencing of ATP7A enhanced this deprivation, further compromising the fungus survival.

Copper is a micronutrient necessary for the activity of enzymes that participate in important processes, such as those related to detoxification [ 39], respiration [8] and capture of metals [13]. Therefore, the absence of copper may compromise the fungus ability to adapt, survive and infect the host [40–42]. Here, through a proteomic approach, we identified differentially expressed proteins related to important processes, beta oxidation, glyoxylate cycle, pentose phosphate pathway and cell wall polymer synthesis. We also identified differentially expressed proteins related to cellular respiration and detoxification.

Metabolic reprogramming by activation of alternative pathway of carbon consumption is a mechanism characterized in *P. brasiliensis* to survive in hostile conditions imposed by the host [43,44]. Our data suggest in copper deprivation, a shift of metabolism towards beta oxidation and glyoxylate cycle, since enzymes for both processes were significantly increased. In this sense, during the infection of macrophages by *P. brasiliensis* glucose availability is low and oxidation of fatty acids is an energy source for the survival of this fungus [43]. In infection in both, interferon-gamma primate or not macrophages, activation of the glyoxylate cycle is a common strategy used by this fungus [44]. The induction of genes of the glyoxylate cycle and beta-oxidation is also described during the infection of *A. fumigatus* conidia in neutrophils [45]. Therefore, the activation of these alternative pathways for energy production may be a mechanism used for *P. brasiliensis* in response to copper deprivation *in vitro* and *in vivo*.

In view of the identification of proteins related to cell wall remodeling, UDP-N-acetylglucosamine pyrophosphorylase (PADG_4312), UDP-galactopyranose mutase (PADG_00912) and 1,4-alpha-glucan-branching enzyme (PADG_12426), we suggest that cell wall remodeling could be occurring in *P. brasiliensis* in copper deprivation. In fact, by fluorescence microscopy, we identified increase of glucans in the cell wall of *P. brasiliensis* in copper deprivation. The remodeling of the cell wall is also described during the infection of macrophages by *P. brasiliensis*, in which there is an increase in the synthesis of glucan and chitin, allowing multiplication and fungal growth within these defense cells [44]. Interestingly, in this condition the synthesis of cell wall polymers was increased and the glucose produced through of the intermediates glyoxylate cycle could be used to remodel the cell wall.

The ability of *P. brasiliensis* in copper depletion to defend against reactive oxygen species seems affected in a critical way. Indeed, our results show that there was an increase in reactive oxygen species in 12 and 24 h of copper deprivation. In addition to the above-mentioned, thioredoxin, GST and Sod2p increased; 6-phosphogluconate dehydrogenase (PADG_03651) related to the production of NADPH via the pentose phosphate pathway also accumulated. NADPH plays an important role in maintaining yeast cell antioxidant activity [48]. Fungi such as *Paracoccidioides lutzii* and *Saccharomyces cerevisiae*, under oxidative stress, use NADPH, produced by the pentose phosphate, due to its reducing power [25,48]. *Pseudomonas fluorescens,* requires NADPH when the organism undergoes oxidative stress [49]. We also identified increase in the chaperone Atx1p, a protein that may act in the response to oxidative stress. This protein which is associated with copper trafficking and iron transport [5,7], presents important role in *S. cerevisiae*, protecting the fungal cell against superoxide anion and hydrogen peroxide toxicity, when Sod1p activity is compromised [5,6].

Although there was a significant increase in some proteins of the respiratory chain, we detected by biochemical assays, that Cox1p activity was strongly reduced during copper deprivation. The role of copper as a cofactor of the enzyme Coxp indicates that changes in the levels of this micronutrient might affect the respiratory process. The presence of this metal ensures the transport of electrons and the reduction of molecular oxygen to water from the respiratory chain complex IV [34]. In *S. cerevisiae*, copper depletion caused by deletion of the gene that codify for the transcription factor *Mac1*, results in reduction in the fungus respiratory capacity [50]. Additionally, an alternative oxidase Aoxp was increased. These results suggest that copper deprivation led to

impaired respiratory activity in *P. brasiliensis* and that positive Aoxp regulation would compensate for this loss. This enzyme is described in plants and fungi as an important component acting as an alternative respiratory route during impairment in Coxp activity, as a redox agent and in cellular homeostasis [34,51,52]. A study carried out with the ascomycete *Podospora anserina* shows that copper plays a fundamental role in the activity of cytochrome c oxidase, and that the deprivation of this metal leads to induction of an alternative pathway, mediated by the alternative oxidase enzyme [53].

Aoxp also functions as a detoxifying agent in mitochondria in the *Paracoccidioides* genus [7,54]. A relationship between the efficiency of the respiratory process, the production of reactive oxygen species (ROS) and role of superoxide dismutase and Aoxp was described [40]. Sod1p (PADG_07418) was decreased in our study, while thioredoxin (PADG_05504), GST (PADG_02526) and Sod2p (PADG_01954) were increased. The superoxide anion formed during the respiratory process, is released in the mitochondrial matrix, by complexes I and III, reacting with manganese and iron dependent mitochondrial superoxide dismutase (Sod2p). The superoxide anion when released to the intermembrane space (IMS), by complex III, reacts with Sod1p. Although Sod1p is a cytoplasmic enzyme, its presence in IMS is described whenever there is a high production of ROS by the respiratory chain process [40,55,56]. Considering the low activity of Sod1p, both Sod2p and Aoxp may act to compensate for the loss of efficiency in the mitochondrial detoxification process and at the same time compensate the respiratory activity of the fungus. Sod1p and Aoxp were also related to the adaptation of *C. albicans* to copper deprivation, in which Aoxp not only compensates for the deficiencies of Coxp activity but also for the loss of Sod1p activity by minimizing mitochondrial ROS [40]. Further, thioredoxin and GST could compensate the decrease in Sod1 activity in the cytosol. Aoxp would be not only compensating for decreased Cox1p activity, but also reducing mitochondrial ROS levels increased by decreased Sod1p expression. A schematic drawing is depicted in (**Figure 8**).

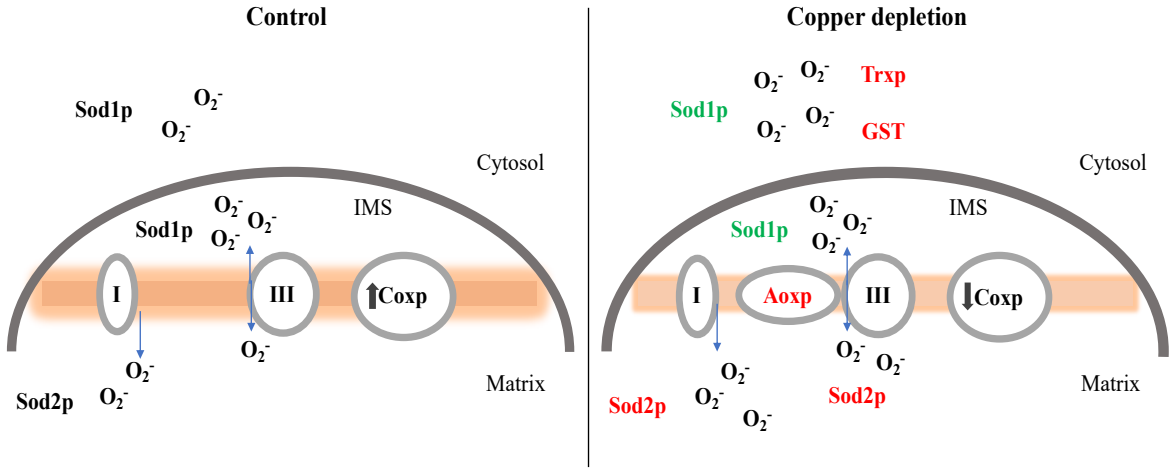


**FIGURE 8.** Changes in cytosolic and mitochondrial protein expression in response to copper deprivation**.** During copper deprivation the decrease of Sod1p in the cytosol and mitochondrial intermembrane space (IMS) as well as the decrease in cytochrome c oxidase (Cox1p) activity, promotes increase in the activity of the alternative oxidase enzyme (Aoxp) as well as an increase in superoxide dismutase Mn/Fe (Sod2p), thioredoxin (Trxp) and glutathione S transferase (GST). Proteins marked red represent the up-regulated and green down-regulated.

In the present study, we propose an overview of the adaptive response of *P. brasiliensis* to copper deprivation, a condition faced by the fungus during infection, at least in macrophages. Metabolic shift towards beta-oxidation and glyoxylate cycle, ROS detoxification and respiration seem to be the most affected processes by the deprivation of this metal. In synthesis, copper deprivation compromised the activity of Sod1p, putatively affecting detoxification, This probably lead to increase in thioredoxin, GST and Sod2p in addition, to up-regulation of the pentose phosphate pathway enzyme, 6-phosphogluconate dehydrogenase, related to the production of NADPH, putatively enhancing the response to the increase in ROS generated by copper deprivation. Also, increase in Atx1 expression could compensate for the decrease in Sod1p expression.

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## AUTHOR CONTRIBUTIONS STATEMENT

CMAS and GP, conceived and designed the experiments. GP, JP, JC, CM and AC, performed the experiments. GP, AB, WF, CR, MP and CMAS analyzed and/or interpreted the data. CMAS contributed to reagents and materials. GP, JC and CMAS analyzed the data and wrote the manuscript.

## CONTRIBUTION TO THE FIELD STATEMENT

During the infective process in the human host, fungi face different defense mechanisms that inhibit the disease progression; one of these mechanisms is the nutritional immunity. In order to survive this condition, pathogens developed strategies, such as expression of the high affinity micronutrient uptake system or cellular remodeling metabolism. In this sense, characterize the adaptive responses in the face of conditions imposed by the host, may facilitate the understanding the survival strategies used by pathogens during the development of diseases. In fungus of the *Paracoccidioides* complex, few studies have characterized the influence of copper on the metabolic adaptation. Thus, this work characterizes, for the first time, the proteomic responses of *P. brasiliensis* to the deprivation of this metal. Data here presented depicted a shift in cellular metabolism, and in response to oxidative stress, in *P. brasiliensis* yeast cells in copper deprivation. Of relevance, a condition faced by the fungi during the infective pathway, can provide new potential targets for drugs against PCM, since copper deprivation, is a condition also found in host, as here demonstrated. Candidate molecules can be Aoxp and Atx1p.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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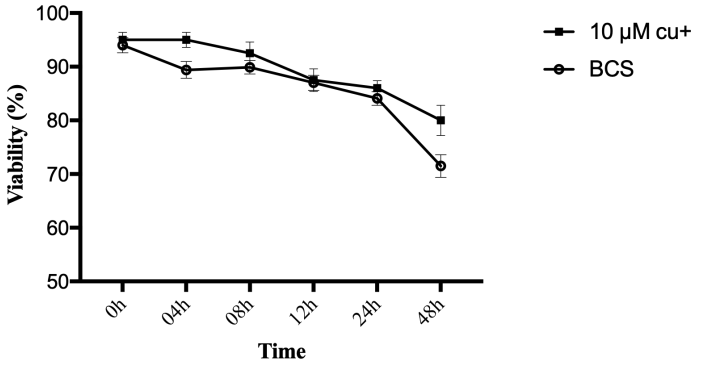
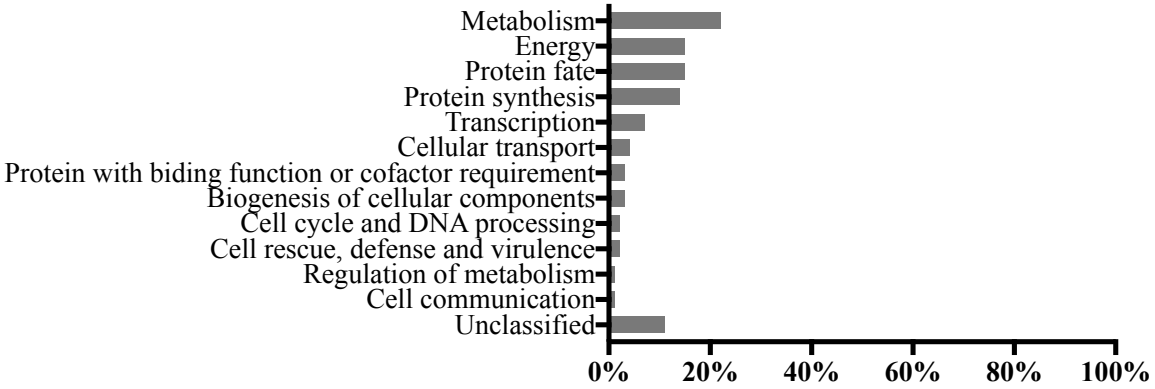
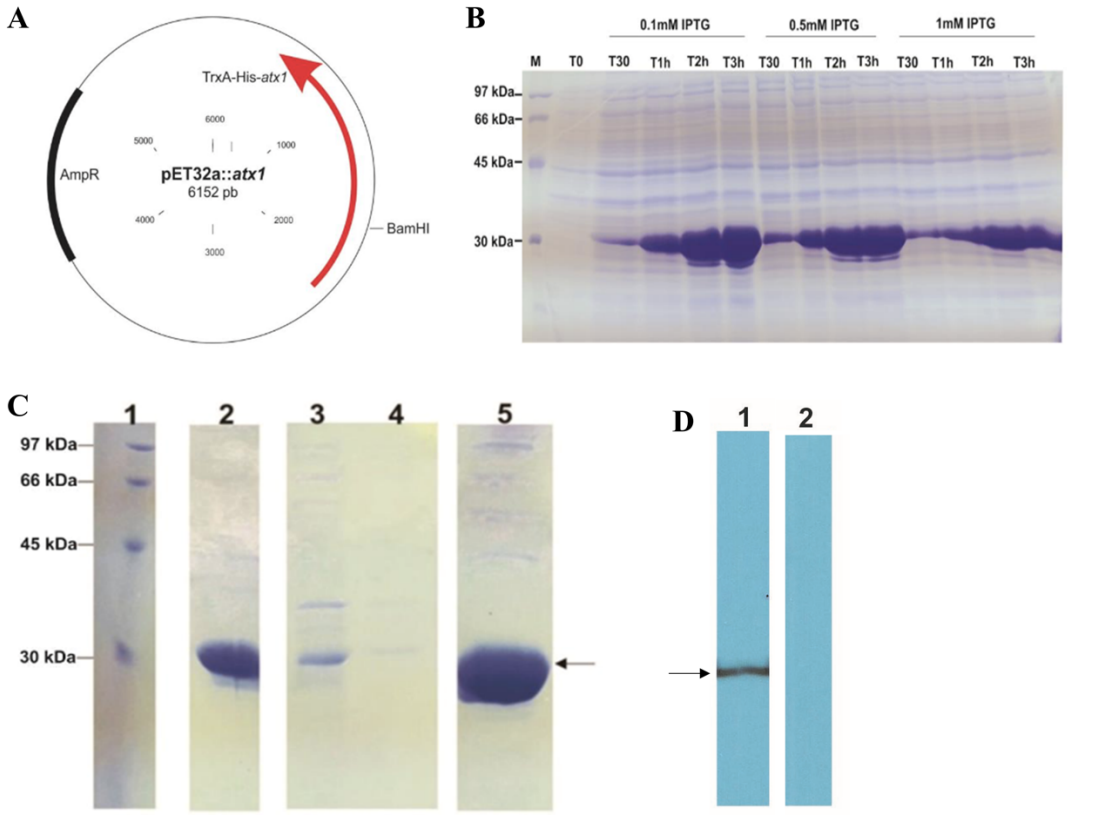
## Supplementary material




**SUPPLEMENTARY FIGURE 1.** Viability of yeast cells of *P. brasiliensis* at time-points in copper deprivation. Copper deprivation was obtained by addition of 50 µM bathocuproinedisulfonic – BCS. Control was obtained by addition of 10 µM of $CuSO_4$.




**SUPPLEMENTARY FIGURE 2.** Functional classification of differentially expressed proteins in yeast cells after 24 h of copper deprivation. All identified proteins were classified according to Functional Catalogue (FunCat2).




**SUPPLEMENTARY FIGURE 3**. Obtaining the recombinant Atx1p. **(A)** PET32a::Atx1 vector. In red the *atx1* gene fused to the histidine tail (trxA-His6-atx1); black Ampicillin resistance gene (AmpR). **(B)** Standardization of induction of recombinant Atx1p with IPTG. M - molecular weight marker; T0- Control; T30- Induction time of 30 min; T1h - Induction time of 1 h; T2h- Induction time of 2 h; T3 - Induction time of 3 h. **(C)** Purification of recombinant Atx1p. The recombinant protein was purified by nickel column affinity chromatography and the material was analyzed on 12% polyacrylamide gel. 1 - Molecular weight marker; 2 - Nickel resin after binding to recombinant Atx1p; 3 - First wash with buffer containing 20 mM imidazole; 4 - Second wash with buffer containing 20 mM imidazole; 5 - Eluted fraction of recombinant Atx1p in buffer containing 250 mM imidazole. The arrow indicates the recombinant Atx1p. **(D)** Analysis of the reactivity of polyclonal anti-Atx1 antibodies by Western blotting. 1- Detection of Atx1p from *P. brasiliensis* yeast cells protein extract using anti-Atx1 antibodies; 2- Negative control - Reactivity of Atx1 with sera of pre-immune animals. The arrow indicates native Atx1p protein.

**Supplementary Table 1** – Oligonucleotides employed in the experiments.

| Gene/Accession Numbers | Oligonucleotides | Tm ($^0$C)* |
|---|---|---|
| L34 Fwd/PADG_04085 | 5'TCAATCTCTCCCGCGAATCC3' | 62 |
| L34 Rev/ PADG_04085 | 5'AGTTGGCGATTGTTGTGCGG3' | 62 |
| CTR3 Fwd/ PADG_05084 | 5'GCTCCTGCGTGATTTCTATGC3' | 60 |
| CTR3 Rev/ PADG_05084 | 5'CTCAAGCGTTTCCCAGCGAG3' | 60 |
| ATX1 Fwd/ PADG_02352 | 5'GGCTGATATCGGATCCATGGCTTCCGTCGAACATC3' | 56 |
| ATX1 Rev/ PADG_02352 | 5'GTGCGGCCGCAAGCTTTCAAACATCCTTCGGCTCGO3' | 56 |

* TM: Melting Temperature

**Supplementary Table 2** - *Paracoccidioides brasiliensis* proteins with increased expression in yeast cells after 24 h of copper depletion.

| GenBank Accession Number [a] | Description[b] | Unique peptides[c] | Fold[d] | Score[e] |
|---|---|---|---|---|
| | **Metabolism** | | | |
| ***Amino acid metabolism*** | | | | |
| PADG_02214 | 4-aminobutyrate aminotransferase | 15 | 1,55 | 12,59 |
| PADG_01797 | Dihydrolipoamide S-acetyltransferase | 2 | 1,49 | 34,726 |
| PADG_03514 | 2-oxoisovalerate dehydrogenase subunit alpha | 5 | 1,44 | 22,906 |
| PADG_03627 | 2-oxoisovalerate dehydrogenase subunit beta | 7 | 1,40 | 15,386 |
| PADG_07369 | Isovaleryl-CoA dehydrogenase | 13 | 1,58 | 12,192 |
| PADG_01621 | Aspartate aminotransferase | 23 | 1,41 | 34,916 |
| PADG_04516 | Glutamate dehydrogenase | 3 | 1,23 | 9,571 |
| PADG_00210 | Glycine dehydrogenase | 20 | 1,63 | 11,515 |
| PADG_08465 | Fumarylacetoacetase | 9 | 1,45 | 45,951 |
| PADG_08466 | Homogentisate 1,2-dioxygenase | 13 | 1,44 | 9,775 |
| PADG_00663 | Homoserine dehydrogenase | 6 | 1,44 | 14,475 |
| PADG_08406 | *O*-acetylhomoserine (thiol)-lyase | 14 | 1,68 | 9,536 |
| PADG_01928 | *S*-adenosylmethionine synthetase | 7 | 1,65 | 14,493 |
| PADG_06252 | 1,2-dihydroxy-3-keto-5-methylthiopentenedioxygenase | 4 | 1,76 | 12,596 |

| | | | | |
|---|---|---|---|---|
| PADG_06289 | Lysine decarboxylase | 3 | 1,29 | 16,68 |
| PADG_00405 | Choline dehydrogenase | 4 | 1,63 | 15,384 |
| PADG_06252 | 1,2-dihydroxy-3-keto-5-methylthiopentenedioxygenase | 4 | 1,76 | 15,564 |
| PADG_07241 | Dihydroxy-acid dehydratase | 6 | 1,40 | 20,631 |
| ***Nitrogen, sulfur and selenium metabolism*** | | | | |
| PADG_02048 | Nitroredutase | 5 | 1,52 | 4,521 |
| PADG_06490 | Formamidase | 14 | 1,40 | 52,093 |
| PADG_00637 | Arginase | 8 | 1,57 | 29,428 |
| PADG_08300 | ornithine transcarbamilase | 6 | 1,48 | 20,304 |
| ***Nucleotide, nucleoside, nucleobase metabolism*** | | | | |
| PADG_00322 | Xantina-fosforibosil-transferase | 5 | 1,36 | 74,748 |
| PADG_07970 | Dihydroorotase | 1 | 1,50 | 7,874 |
| PADG_05812 | CTP synthase | 1 | 1,31 | 5,767 |
| ***Phosphate metabolism*** | | | | |
| PADG_04175 | Inorganic pyrophosphatase | 2 | 1,29 | 10,732 |
| ***Biosynthesis of vitamins, cofactors and prosthetics groups*** | | | | |
| PADG_01886 | Adenosyl homocysteinase | 23 | 1,53 | 10,582 |
| PADG_05822 | Pyridoxine biosynthesis | 4 | 1,45 | 75,16 |
| ***C-compound and carbohydrate metabolism*** | | | | |
| PADG_01486 | Short chain dehydrogenase/reductase family | 2 | 1,29 | 22,341 |
| PADG_07618 | Succinate-semialdehyde dehydrogenase | 1 | 1,64 | 6,473 |
| PADG_00604 | Phosphoacetylglucosamine mutase | 3 | 1,56 | 108,085 |
| PADG_03671 | Phenylpyruvate tautomerase | 4 | 1,55 | 28,847 |
| PADG_06199 | FAD binding domain-containing protein | 5 | 1,48 | 30,384 |
| ***Lipid, fatty acid, and isoprenoid metabolism*** | | | | |
| PADG_00254 | Fatty acid synthase subunit alpha reductase | 32 | 1,40 | 9,621 |
| PADG_05904 | Dihydrolipoamide succinyltransferase | 7 | 1,48 | 9,486 |

| | | | | |
|---|---|---|---|---|
| PADG_00678 | Oxiterol protein | 2 | 1,77 | 5,602 |
| ***Metabolism of vitamins, cofactors, and protetics group*** | | | | |
| PADG_00443 | Dihydropteroate synthase | 10 | 1,60 | 45,283 |
| PADG_00607 | Riboflavin synthase alpha chain | 2 | 1,48 | 20,304 |
| **Energy** | | | | |
| ***Glycolysis and gluconeogenesis*** | | | | |
| PADG_00852 | Fructose 1,6, biphosphate aldolase | 2 | 1,64 | 5,938 |
| PADG_05109 | Phosphoenolpyruvate carboxykinase | 20 | 1,52 | 54,408 |
| PADG_01706 | Fructose-1,6-bisphosphatase | 7 | 2,57 | 28,593 |
| PADG_02411 | Glyceraldehyde-3-phosphate dehydrogenase | 26 | 1,31 | 51,116 |
| PADG_05081 | Aldehyde dehydrogenase | 14 | 1,52 | 54,187 |
| ***Fermentation*** | | | | |
| PADG_00714 | Pyruvate decarboxylase | 15 | 2,56 | 64,219 |
| ***Glyoxylate cycle*** | | | | |
| PADG_04709 | Isocitrate lyase | 9 | 1,43 | 23,482 |
| PADG_11845 | Aconitate hydratase | 37 | 1,32 | 14,452 |
| PADG_08059 | Malate dehydrogenase | 16 | 1,65 | 12,985 |
| ***Citric acid cycle*** | | | | |
| PADG_04249 | Isocitrate dehydrogenase | 16 | 1,60 | 12,989 |
| PADG_06494 | Dihydrolipoyl dehydrogenase | 18 | 1,42 | 44,83 |
| ***Pentose-phosphate pathway*** | | | | |
| PADG_03651 | 6-phosphogluconate dehydrogenase | 15 | 1,54 | 9,235 |
| PADG_04604 | Transketolase | 17 | 1,50 | 40,523 |
| ***Electron transport and membrane-associated energy conservation*** | | | | |
| PADG_11468 | NADPH-dependent diflavin oxidoreductase 1 | 3 | 1,29 | 32,277 |
| PADG_05523 | Quinone oxidoreductase | 4 | 1,46 | 33,41 |
| PADG_11302 | VosA protein | 2 | 1,48 | 28,593 |

| | | | | |
|---|---|---|---|---|
| ***Respiration*** | | | | |
| PADG_05905 | NADH dehydrogenase | 3 | 1,51 | 132,419 |
| PADG_11605 | NADPH-cytochrome P450 reductase | 13 | 1,67 | 48,746 |
| PADG_08349 | ATP synthase beta subunit | 23 | 1,45 | 43,125 |
| PADG_07081 | Electron transfer flavoprotein subunit alpha | 4 | 1,59 | 5,701 |
| PADG_03747 | Alternative oxidase | 2 | 2,37 | 48,917 |
| PADG_03559 | Cytochrome b5 | 3 | 1,35 | 54,408 |
| ***Oxidation of fatty acids*** | | | | |
| PADG_00382 | Acetyl-CoA acyltransferase | 16 | 1,50 | 12,314 |
| PADG_01687 | Acetyl-CoA acyltransferase | 18 | 1,50 | 54,56 |
| PADG_02852 | Acyl-CoA dehydrogenase | 12 | 1,57 | 23,975 |
| PADG_07023 | Carnitine O-acetyltransferase | 9 | 1,49 | 45,439 |
| **Cell cycle and DNA processing** | | | | |
| ***DNA processing*** | | | | |
| PADG_05798 | Single-strand binding protein family | 8 | 1,51 | 12,998 |
| PADG_02683 | UV excision repair protein Rad23 | 4 | 1,50 | 9,479 |
| PADG_00656 | Non-histone chromosomal protein 6 | 3 | 1,57 | 8,972 |
| **Transcription** | | | | |
| ***mRNA synthesis*** | | | | |
| PADG_02555 | Nucleic acid-binding protein | 14 | 1,51 | 65,114 |
| PADG_03836 | Heterogeneous nuclear ribonucleoprotein A1 | 1 | 1,62 | 12,221 |
| PADG_08717 | Splicing factor 3B subunit 4 | 18 | 1,41 | 40,476 |
| PADG_05190 | Coativador RNA polimerase II | 2 | 1,37 | 24,671 |
| ***Transcriptional control*** | | | | |
| PADG_00873 | Histone H3 | 5 | 1,33 | 29,329 |
| PADG_05906 | Histone H2A | 2 | 1,67 | 5,864 |
| PADG_02410 | Histone deacetylase hda1 | 2 | 1,39 | 5,938 |

| | | | | |
|---|---|---|---|---|
| PADG_07134 | Histone H4 | 1 | 1,41 | 73,285 |
| ***Transcription repression*** | | | | |
| PADG_06182 | Transcriptional repressor TUP1 | 7 | 1,46 | 25,825 |
| ***RNA processing*** | | | | |
| PADG_00041 | Ran gtpase-activating protein | 4 | 1,40 | 11,807 |
| PADG_05393 | mRNA decapping hydrolase | 2 | 1,38 | 10,141 |
| ***Splicing*** | | | | |
| PADG_01892 | Pre-mRNA-processing factor 40 | 1 | 1,52 | 4,82 |
| **Protein synthesis** | | | | |
| ***Ribosome biogenesis*** | | | | |
| PADG_01407 | 40S ribosomal protein | 21 | 1,70 | 30,995 |
| PADG_00335 | 40S ribosomal protein S14 | 10 | 1,45 | 20,335 |
| PADG_04118 | 60S ribosomal protein L38 | 4 | 1,80 | 43,681 |
| PADG_06680 | 40S ribosomal protein S22 | 7 | 1,54 | 14,578 |
| PADG_07863 | 40S ribosomal protein S8 | 6 | 1,68 | 2,121 |
| PADG_04315 | 40S ribosomal protein L24 | 10 | 1,63 | 6,078 |
| PADG_12365 | 40S ribosomal protein S8 | 8 | 1,66 | 6,114 |
| PADG_02828 | 60S ribosomal protein l10a | 8 | 1,50 | 23,192 |
| PADG_02888 | 60S ribosomal protein L6 | 10 | 1,46 | 14,08 |
| PADG_03778 | 60S ribosomal protein L10-A | 9 | 1,58 | 12,002 |
| PADG_03873 | 60S ribosomal protein L20 | 8 | 1,57 | 9,089 |
| PADG_04065 | 60S ribosomal protein L36 | 4 | 1,48 | 23,125 |
| PADG_04402 | 60S ribosomal protein L34-A | 3 | 1,66 | 32,009 |
| PADG_12253 | 60S ribosomal protein L3 | 20 | 1,55 | 47,14 |
| PADG_05836 | 60S ribosomal protein L39 | 1 | 1,61 | 65,681 |
| PADG_04449 | 60S ribosomal protein l23e | 5 | 1,47 | 10,876 |
| PADG_05338 | 60S ribosomal protein L18 | 7 | 1,51 | 7,342 |

| | | | | |
|---|---|---|---|---|
| PADG_05883 | 60S ribosomal protein l23a | 7 | 1,52 | 15,12 |
| PADG_06568 | 60S ribosomal protein L23 | 8 | 1,32 | 3,456 |
| PADG_08213 | 40S ribosomal protein S0 | 6 | 1,60 | 32,29 |
| PADG_07864 | 40S ribosomal protein S8 | 6 | 1,68 | 17,10 |
| ***Translation*** | | | | |
| PADG_6265 | Elongation factor 1-gamma | 28 | 1,45 | 6,845 |
| PADG_8125 | Elongation fator 2 | 36 | 1,70 | 3,224 |
| ***Aminoacyl-tRNA-synthetases*** | | | | |
| PADG_1558 | Histidyl-tRNA synthetase | 11 | 1,64 | 5,246 |
| PADG_02918 | Prolyl-tRNA synthetase | 4 | 1,40 | 6,481 |
| PADG_03440 | Prolyl-tRNA synthetase | 6 | 1,37 | 14,578 |
| **Protein fate** | | | | |
| ***Protein folding and stabilization*** | | | | |
| PADG_00001 | Peptidyl-prolyl cis-trans isomerase H | 6 | 1,63 | 21,878 |
| PADG_00207 | Hsp40 Head shock protein | 4 | 1,77 | 13,174 |
| PADG_00430 | Hsp7 protein | 35 | 1,57 | 2,738 |
| PADG_03562 | Hsp70 protein | 32 | 1,44 | 35,185 |
| PADG_05094 | T-complex protein 1 subunit zeta | 38 | 1,54 | 34,681 |
| PADG_08369 | Hsp60-like protein | 43 | 1,54 | 27,583 |
| ***Protein targeting, sorting and translocation*** | | | | |
| PADG_03882 | Mitochondrial import inner membrane translocase | 3 | 1,40 | 29,394 |
| ***Protein modification*** | | | | |
| PADG_02637 | Ubiquitin conjugating enzyme | 4 | 1,59 | 14,332 |
| ***Modification by phosphorylation, dephosphorylation, autophosphorylation*** | | | | |
| PADG_02212 | Serine/threonine-protein phosphatase | 5 | 1,34 | 3,951 |
| PADG_03001 | N-terminal acetyltransferase A complex catalytic | 1 | 1,44 | 19,220 |
| PADG_07925 | Ubiquitin-conjugating enzyme | 2 | 1,65 | 3,191 |

| | | | | |
|---|---|---|---|---|
| PADG_05245 | Ubiquitin-NEDD8-like protein RUB2 | 2 | 1,62 | 31,053 |
| ***Protein processing (proteolytic)*** | | | | |
| PADG_01992 | Mitochondrial-processing peptidase subunit alpha | 2 | 1,45 | 26,413 |
| PADG_06766 | Mitochondrial-processing peptidase subunit beta | 8 | 1,35 | 12,076 |
| ***Assembly of protein complex*** | | | | |
| PADG_05335 | Iron sulfur cluster assembly protein | 5 | 1,44 | 3,13 |
| PADG_07964 | Vacuolar ATP synthase subunit E | 4 | 1,56 | 22,858 |
| ***Protein/peptide degradation*** | | | | |
| PADG_00071 | 26S proteasome regulatory subunit RPN10 | 1 | 1,76 | 3,361 |
| PADG_04167 | Aspartyl aminopeptidase | 10 | 1,57 | 2,715 |
| PADG_05922 | Cytosolic non-specific dipeptidase | 10 | 1,54 | 6,216 |
| PADG_06290 | Proteossome endopeptidase complex | 4 | 1,48 | 2,953 |
| ***Cytoplasmic and nuclear protein degradation*** | | | | |
| PADG_08442 | Proteasome component Y13 | 8 | 1,37 | 16,991 |
| ***Proteasomal degradation (ubiquitin/proteasomal pathway)*** | | | | |
| PADG_02735 | Proteasome component PRE6 | 4 | 1,38 | 9,734 |
| PADG_03680 | Proteasome component PRE2 | 6 | 1,51 | 11,433 |
| PADG_03982 | Proteasome component C1 | 12 | 1,40 | 2,715 |
| ***Lysosomal and vacuolar protein degradation*** | | | | |
| PADG_00634 | Vacuolar protease A | 1 | 1,70 | 14,996 |
| **Protein with biding function or cofator requirement** | | | | |
| ***DNA binding*** | | | | |
| PADG_04311 | Cellular nucleic acid-binding protein | 5 | 1,39 | 21,911 |
| ***RNA binding*** | | | | |
| PADG_07884 | Polyadenylate-binding protein | 21 | 1,39 | 4,573 |
| ***ATP binding*** | | | | |
| PADG_06294 | Hsp70 nucleotide exchange factor fes1 | 1 | 1,37 | 9,049 |
| ***Vesicular transport (Golgi network, etc.)*** | | | | |

| | | | | |
|---|---|---|---|---|
| PADG_03392 | GTP-binding protein ypt5 | 1 | 1,40 | 5,395 |
| ***GTP binding*** | | | | |
| PADG_00172 | Ras-like GTP-binding protein | 1 | 1,40 | 5,384 |
| PADG_04048 | Small COPII coat GTPase sar1 | 2 | 1,74 | 35,844 |
| **Regulation of metabolism** | | | | |
| ***Regulation by modification*** | | | | |
| PADG_04420 | Peptide methionine sulfoxide reductase msra | 1 | 1,59 | 4,453 |
| ***Enzyme inhibitor*** | | | | |
| PADG_01891 | Translation initiation factor RLI1 | 6 | 1,72 | 15,238 |
| **Cellular transport** | | | | |
| PADG_00044 | Nucleolin (492 a | 2 | 1,38 | 6,188 |
| PADG_01847 | Nucleolin (452 aa) | 3 | 1,42 | 8,972 |
| PADG_11111 | Golgi apparatus membrane protein | 1 | 1,44 | 5,654 |
| PADG_02352 | Copper chaperone ATX1 | 2 | 1,86 | 16,890 |
| PADG_03562 | Chaperone Dnak | 34 | 1,66 | 12,345 |
| PADG_07014 | Vesicular-fusion protein sec17 | 5 | 1,47 | 10,029 |
| **Cell communication** | | | | |
| ***Cellular signaling*** | | | | |
| PADG_01530 | G-protein comlpex beta subunit cpcb | | | |
| **Cell rescue, defence and virulence** | | | | |
| ***Oxidative stress response*** | | | | |
| PADG_05504 | Thioredoxin | 8 | 1,59 | 39,381 |
| PADG_01954 | Superoxide dismutase Mn/Fe dependente | 7 | 1,86 | 20,034 |
| PADG_02526 | Glutathione S transferase | 2 | 1,83 | 4,573 |
| **Biogenesis of cellular componentes** | | | | |
| ***Cell wall*** | | | | |
| PADG_04312 | UDP-N-acetylglucosamine pyrophosphorylase | 6 | 1,38 | 12,989 |

| | | | | |
|---|---|---|---|---|
| PADG_00912 | UDP-galactopyranose mutase | 12 | 1,49 | 7,898 |
| PADG_12426 | 1,4-alpha-glucan-branching enzyme | 5 | 1,48 | 3,454 |
| ***Actin cytoskeleton*** | | | | |
| PADG_05538 | Actin | 4 | 1,42 | 35,594 |
| PADG_12077 | Actin | 11 | 1,43 | 37,732 |
| **Unclassified** | | | | |
| PADG_00440 | Hypothetical protein | 6 | 1,55 | 12,52 |
| PADG_00676 | Hypothetical protein | 5 | 1,37 | 8,788 |
| PADG_01343 | Hypothetical protein | 6 | 1,55 | 2,367 |
| PADG_02342 | Hypothetical protein | 7 | 1,60 | 9,589 |
| PADG_01867 | Hypothetical protein | 12 | 1,67 | 6,895 |
| PADG_03203 | Hypothetical protein | 7 | 1,47 | 7,126 |
| PADG_03244 | Hypothetical protein | 3 | 1,34 | 17,104 |
| PADG_03886 | Hypothetical protein | 1 | 1,23 | 2,367 |
| PADG_04439 | Hypothetical protein | 6 | 1,59 | 13,792 |
| PADG_04636 | Hypothetical protein | 11 | 1,45 | 18,998 |
| PADG_06488 | Hypothetical protein | 19 | 1,46 | 30,762 |
| PADG_07264 | Hypothetical protein | 3 | 1,50 | 43,141 |
| PADG_07627 | Hypothetical protein | 2 | 1,43 | 33,464 |
| PADG_07670 | Hypothetical protein | 3 | 1,39 | 30,041 |
| PADG_07836 | Hypothetical protein | 5 | 2,79 | 35,185 |
| PADG_08037 | Hypothetical protein | 2 | 1,48 | 15,238 |
| PADG_08283 | Hypothetical protein | 1 | 1,22 | 65,906 |
| PADG_08715 | Hypothetical protein | 9 | 1,59 | 15,164 |
| PADG_08605 | Hypothetical protein | 1 | 1,66 | 9,54 |
| PADG_02981 | Hypothetical protein | 4 | 1,29 | 11,319 |

[a,b)] Accession Number and description of protein according to Genbank NCBI and MIPS database respectively:
Genbank NCBI: https://www.ncbi.nlm.nih.gov/genbank/ and Pedant on MIPS-Functional Catalogue (http://pedant.helmholtz-muenchen.de/);[c, d)] indicates the number of peptides identified for a given protein and the fold change value..

**Supplementary Table 3 -** *Paracoccidioides brasiliensis* proteins with decreased expression in yeast cells after 24 h of copper depletion.

| GenBank Accession Number [a] | Description[b] | Unique peptides[c] | Fold[d] | Score |
|---|---|---|---|---|
| | **Metabolism** | | | |
| PADG_06332 | Pro-apoptotic serine protease | 2 | 0,78 | 12,776 |
| PADG_04293 | adenine phosphoribosyltransferase | 3 | 0,83 | 9,265 |
| | **Energy** | | | |
| PADG_12145 | Uracil-regulated protein 1 | 4 | 0,81 | 3,341 |
| PADG_12144 | GTP cyclohydrolase | 2 | 0,77 | 21,078 |
| | **Protein fate** | | | |
| PADG_07190 | Proteasome endopeptidase complex | 3 | 0,79 | 16,155 |
| PADG_02212 | Serine/threonine-protein phosphatase | 4 | 0,80 | 6,019 |
| | **Cellular transport** | | | |
| PADG_07804 | Protein transporter SEC23 | 2 | 0,83 | 8,991 |
| PADG_02640 | ABC transporter ATP-binding protein ARB1 | 1 | 0,77 | 3,467 |
| | **Cell rescue, defence and virulence** | | | |
| PADG_07418 | Superoxide dismutase Cu-Zn | 5 | 0,63 | 20,033 |
| | **Unclassified** | | | |
| PADG_01900 | Hypothetical protein | 3 | 0,78 | 14,818 |

[a,b)] Accession Number and description of protein according to Genbank NCBI and MIPS database respectively:
Genbank NCBI: https://www.ncbi.nlm.nih.gov/genbank/ and Pedant on MIPS-Functional Catalogue (http://pedant.helmholtz-muenchen.de/); [c, d)] indicates the number of peptides identified for a given protein and the fold change value.

# Capítulo III

## 1. Discussão

O cobre é um micronutriente necessário para a atividade de enzimas que participam de processos importantes, como os relacionados à desintoxicação (PEÑA; KOCH; THIELE, 1998), respiração (HORNG et al., 2004) e captura de metais como ferro (GEORGATSOU et al., 1997). Portanto, um eficiente sistema de homeostase desse metal é importante para sobrevivência de microorganismo. Durante a infecção do hospedeiro, os fungos patogênicos podem encontrar condições diferentes em relação à concentração de cobre, dependendo do tecido infectado. *Cryptococcus neoformans*, por exemplo, encontra altos níveis de cobre nos pulmões no início da infecção e níveis reduzidos desse metal durante a infecção no cérebro (SUN et al., 2014). Dessa forma, inicialmente buscamos entender qual a condição encontrada pelo *P. brasiliensis* durante a infecção em macrófagos, em relação à concentração de cobre. A partir do silenciamento de uma bomba transportadora de cobre, ATPase do tipo P (ATP7A), presentes em macrófagos e responsável por bombear cobre para fagolisossomos durante a infecção (WIEMANN et al., 2017), verificamos que uma menor quantidade de células foram recuperadas em relação à infecção em macrófagos não silenciados para ATP7A. Os resultados sugerem que o fungo é privado de cobre no interior dos macrófagos e que o silenciamento da ATP7A aumentou essa privação, comprometendo ainda mais a sobrevivência de *P. brasiliensis* dentro dessa célula de defesa.

Em seguida, realizamos uma análise proteômica, baseado em iTRAQ, que nos permitiu propor um *overview* dos processos metabólicos regulados após o cultivo de *P. brasiliensis* em meio privado de cobre. O *overview* apresenta que a privação de cobre levou a um comprometimento nos processos de detoxificação e respiração e a uma mudança no fluxo do metabolismo de carbono, obtenção de energia e remodelamento de parede.

No primeiro caso, relacionado ao comprometimento nos processod de detoxificação e respiração, os resultados sugerem que a baixa concentração de cobre tenha comprometido a atividade da enzima Sod1p (PADG_07418), afetando o processo de desintoxicação, o que levou a um aumento na tioredoxina (PADG_05504), GST (PADG_02526) e Sod2p (PADG_01954). Além disso, a regulação positiva das enzimas da via da pentose fosfato, 6-fosfogluconato desidrogenase (PADG_03651) e Transketolase (PADG_04604) relacionadas à produção de NADPH, bem como um aumento na atividade da acompanhante Atx1p (PADG_02352), ambos relacionados à

desintoxicação reforça essa idéia. Ainda, a up-regulação de uma alternativa oxidase (Aoxp) indicou uma possível alteração na atividade da cadeia respiratória.

A idenficação no aumento da expressão da enzima Aoxp (PADG_03747) e seu papel tanto na detoxificação mitocondrial, quanto como na compensação da diminuição da atividade respiratória, foi considerada um ponto chave para que pudéssemos investigar o papel dessa enzima na adaptação do fungo à privação de cobre. Essa enzima é descrita em plantas e fungos como um componente importante que atua na via respiratória alternativa durante situações em que há comprometimento da atividade da Citocromo c oxidase (Coxp), bem como quando há aumento no número de espécies reativas de oxigênio (JOSEPH-HORNE; HOLLOMON; WOOD, 2001; MARTINS et al., 2011; VISHWAKARMA et al., 2015). Tanto a enzima Coxp, quanto a enzima Sod1p são dependentens de cobre. Por meio de teste enzimático e microscopia de fluorescência identificamos que a atividade da enzima Coxp estava diminuída e que a atividade mitocondrial estava reduzida durante a privação de cobre. Estes dados podem ser observados na figura 6 do artigo apresentado no capítulo 2 desta tese. Baseados nestes resultados, somando o aumento na expressão de tioredorina, GST e Sod2p, hipotetizamos que Aoxp poderia estar desempenhando um papel importante na sobrevivência de *P. brasiliensis* durante a privação de cobre. A relação entre a eficiência do processo respiratório, a produção de espécies reativas de oxigênio (ERO) e o papel da superóxido dismutase e do Aoxp em *Candida albicans* já havia sido descrita descrita (BROXTON; CULOTTA, 2016). O ânion superóxido é um produto formado durante o processo respiratório, sendo liberado na matriz mitocondrial pelos complexos I e III, onde reage com a superóxido dismutase mitocondrial dependente de manganês e ferro (Sod2p) e no espaço intermembranar (IMS), por complexo III, onde reage com Sod1p. Embora o Sod1p seja uma enzima citoplasmática, sua presença no IMS é descrita sempre que há uma maior produção de ERO pelo processo da cadeia respiratória (BROXTON; CULOTTA, 2016; KAWAMATA; MANFREDI, 2010; TURRENS; BOVERIS, 1980). Considerando estas informações, sugerimos que a enzima Aoxp estaria atuando tanto compensando a diminuição na atividade da Coxp, quanto diminuindo o efeito pelo aumento de EROs mitocondrial. Esses dados corroboram um estudo em que o papel das enzimas Sodp e Aoxp na adaptação do fungo Candida albicans à privação de cobre. Nesse estudo, os autores fornecem evidências de que o Aoxp não apenas compensa as deficiências da atividade de Coxp, como também compensa a perda da atividade de Sod1p, minimizando as ERO mitocondriais (BROXTON; CULOTTA, 2016). Diante

disso, propomos um modelo que demonstra as mudanças no perfil proteico citosolico e mitocondrial frente a adaptação à privação de cobre por *P. brasiliensis* que é apresentado na figura 08 presente no artigo do capítulo 2 dessa tese.

Em relação às alterações no metabolismo celular notamos que houve uma mudança no fluxo do metabolismo de carbono, favorecendo a gliconeogênese e culminando no remodelamento de parde. O aumento na expressão de enzimas reguladoras da gliconeogênese, como a fosfoenolpiruvato carboxiquinase (PADG_05109) e a frutose-1,6-bifosfatase (PADG_01706), foram identificadas. Enzimas que degradam aminoácidos gluconeogênicos, como a glicina desidrogenase (PADG_00210), que converte glicogênio por glioxilato, a glutamato desidrogenase (PADG_04516), que por desminação, converte glutamato em α-cetoglutarato e aspartato aminotransferato aspartato (PADG_01621). Além disso, a regulação positiva de enzimas relacionadas à β-oxidação, fornecendo estoques de enzimas do ciclo acetil-CoA e glioxilato, atuaria em favor da gliconeogênese. Tendo em vista a identificação de proteínas relacionadas à remodelação da parede, a UDP-N-acetilglucosamina pirofosforilase (PADG_4312), a UDP-galactopiranose mutase (PADG_00912) e a enzima ramificação da 1,4-alfa-glucana (PADG_12426), hipotetizamos que a gliconeogênese poderia estar ocorrendo em *P. brasiliensis* sob privação de cobre com o intuito de promover um remodelamento de perade. De fato, por microscopia de fluorescência, identificamos um aumento de glucanos na parede celular do fungo cultivado sob privação desse metal em relação ao controle, conforme demonstrado na figura 05 do material apresentado no capítudo 2 desta tese.

## 2. Conclusão

- *Paracoccidioides brasiliensis* responde expressa a proteína Ctr3p sob condição de privação de cobre;
- Para análise proteômica o melhor tempo de cultivo, determinado a partir da análise da expressão de Ctr3 sob privação de cobre e por ançalise de viabilidade por citometria de fluxo, foi de 24 horas;
- Os resultados sugrem que *P. brasiliensis* sofre privação de cobre no interior de macrófagos;
- A partir da análise proteômica e da validação dos resultados analisados, identificamos que *P. brasiliensis* sofre um remodelamento metabólico durante a privação de cobre;
- A privação de cobre afeta os processo de respitaração e a capacidade de detoxificação de *P. brasilisensis* e os resultados obtidos sugerem que este fato tenha levado a mudanças no expressão de proteínas mitocondriais e citosólicas com intuito de restabelecer a homeostase fúngicas.

## 3. Perspectivas

- Caracterizar a maquinaria de homeostase de cobre em fungos do gênero *Paracoccidioides*;
- Ampliar os experimentos acerca da resposta de *P. brasiliensis* durante a infecção em macrófagos, avaliando a expressão de proteínas de homeostase de cobre;
- Avaliar a resposta de *P. brasiliensis* mutado para a proteína alternativa oxidase (Aoxp) durante a privação de cobre;
- Promover uma análise comparativa entre *P. brasiliensis* e outras espécies filogenéticas do gênero *Paracoccidioides* durante a privação de cobre.

## 4. Demais participantes do projeto.

Participa deste projeto como coordenadora da pesquisa a Drª. Célia Maria de Almeida Soares, do Instituto de Ciências Biológicas da UFG. O estudo é realizado em colaboração com o Programa de Patologia Molecular da Universidade de Brasília, com contribuição dos professores Dr. Carlos André Ornelas Ricart e Dr. Wagner Fontes e do Doutor Agenor de Castro Moreira. Ainda, o estudo desenvolvido até o presente momento, teve a contribuição do Dr. Alexandre Bailão, professor do Programa de Genética e Biologia Molecular da Universidade Federal de Goiás (PGBM-UFG), bem como da Drª. Lilian Cristiane Baeza e Drª. Patrícia Lima, ex-bolsistas do PGBM-UFG.

Ainda, toda equipe do laboratório de biologia molecular (LBM), professores e alunos que, direta ou indiretamente, contribuem com suporte, ideias e apoio necessário para que os desafios sejam superados e os resultados alcançados.

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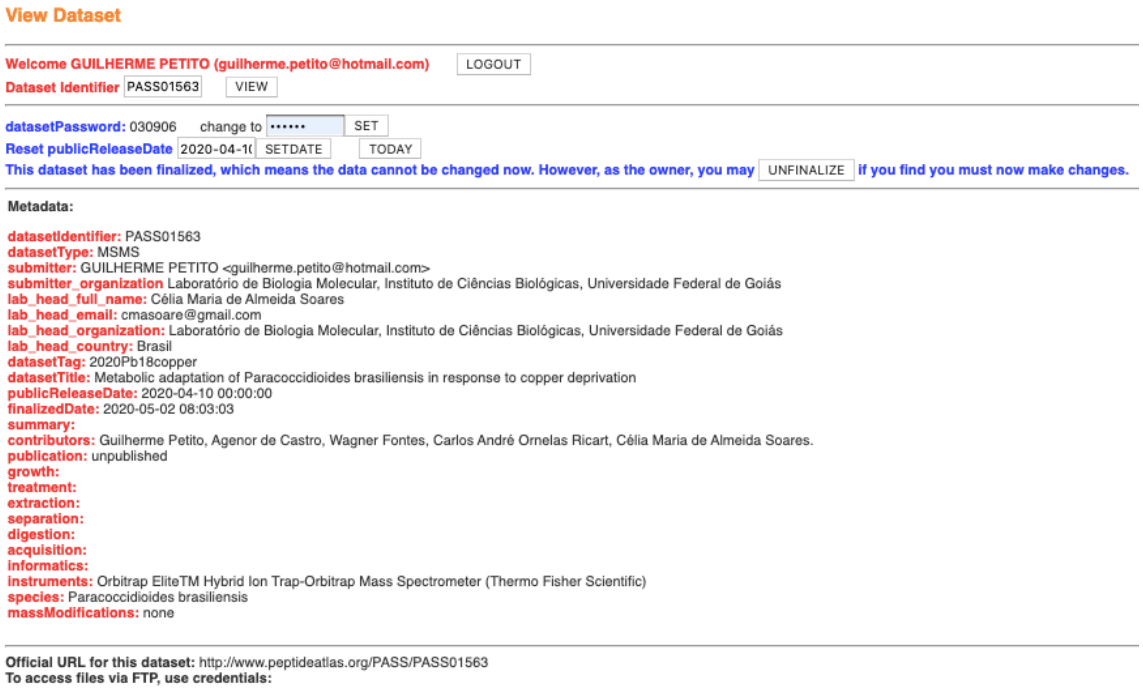
# Anexo

Anexo I

Os arquivos raw do proteôma deste estudo foram depositados na base Peptide Atlas e podem ser acessados pelo código PASS01563, pelo site http://www.peptideatlas.org/



View Dataset

Welcome GUILHERME PETITO (guilherme.petito@hotmail.com) LOGOUT
Dataset Identifier PASS01563 VIEW

datasetPassword: 030906 change to •••••• SET
Reset publicReleaseDate 2020-04-1( SETDATE TODAY
This dataset has been finalized, which means the data cannot be changed now. However, as the owner, you may UNFINALIZE if you find you must now make changes.

Metadata:

datasetIdentifier: PASS01563
datasetType: MSMS
submitter: GUILHERME PETITO <guilherme.petito@hotmail.com>
submitter_organization Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás
lab_head_full_name: Célia Maria de Almeida Soares
lab_head_email: cmasoare@gmail.com
lab_head_organization: Laboratório de Biologia Molecular, Instituto de Ciências Biológicas, Universidade Federal de Goiás
lab_head_country: Brasil
datasetTag: 2020Pb18copper
datasetTitle: Metabolic adaptation of Paracoccidioides brasiliensis in response to copper deprivation
publicReleaseDate: 2020-04-10 00:00:00
finalizedDate: 2020-05-02 08:03:03
summary:
contributors: Guilherme Petito, Agenor de Castro, Wagner Fontes, Carlos André Ornelas Ricart, Célia Maria de Almeida Soares.
publication: unpublished
growth:
treatment:
extraction:
separation:
digestion:
acquisition:
informatics:
instruments: Orbitrap EliteTM Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific)
species: Paracoccidioides brasiliensis
massModifications: none

Official URL for this dataset: http://www.peptideatlas.org/PASS/PASS01563
To access files via FTP, use credentials: