UNIVERSITY OF BRASÍLIA
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GRADUATE PROGRAM IN MOLECULAR PATHOLOGY

Combined strategies for *Cryptococcus* sp. growth control: antifungal drugs, epigenetic modulators, photodynamic therapy and actinobacteria-derived metabolites

KUNAL RANJAN

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Doctoral Thesis presented to the Graduate Program in Molecular Pathology, Faculty of Medicine, University of Brasilia, as part of the requirements to obtain the title of Doctor in Molecular Pathology.

Supervisor: Prof. Dr Marcio José Poças Fonseca

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I dedicate this work to my grandparents, parents, brother and all my family members for the support, encouragement and unconditional affection

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List of publications and conference attended

1) Original articles published

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3) Article and book chapters under review and in preparation

Fernanda Fonsêca Ferreira, **Kunal Ranjan**, Camila Gomes, Rayssa Karla Oliveira, Larissa Fernandes and Marcio José Poças Fonseca. Characterization of *Cryptococcus neoformans*'s putative ATL1 gene. (in preparation)

Kunal Ranjan, Daniel Reis Maiolino de Mendonça, Fernando Pacheco Rodrigues, Mandeep Dixit, Pratyoosh Shukla and Marcio José Poças-Fonseca Antifungal potential of actinobacteria isolated from Indian Traditional Medicine plants against *Cryptococcus* spp. and filamentous fungi isolated from subterranean iron ore plateau in the Carajás National Forest, Brazil. (in preparation)

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Kunal Ranjan, Camila Suguiura, José AV Morais, Dileep Kumar, Marcio José Poças-Fonseca. Evaluation of the interaction amongst antifungal drugs, epigenetic modulators and

photodynamic therapy in *C. neoformans*. 17th Infocus and 1st ISHAM LATAM Congress, 13-16 November, 2019, Salvador, Bahia, Brazil (Poster presentation).

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ABSTRACT

Cryptococcus is a globally distributed fungal pathogen that primarily afflicts immunocompromised individuals. The therapeutic options are limited and include mostly amphotericin B or fluconazole, alone or in combination. The extensive employment of antifungals allowed the selection of resistant pathogens, posing threats to global public health. Histone deacetylase genes are involved in *Cryptococcus neoformans* virulence, and in pathogenicity and resistance to azoles in *Candida albicans*. In this context, in Chapter I of this work, we evaluated the activity of amphotericin B, fluconazole, sulfamethoxazole, sodium butyrate or trichostatin A (histone deacetylase inhibitors), and hydralazine or 5- aza-2′ -deoxycytidine (DNA methyltransferase inhibitors) alone or in combinations against *C. neoformans* H99 (reference), T1 and 89-610 (fluconazole resistant), histone deacetylase null mutant strains (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ and *hda1*Δ+HDA1) and also against *C. gattii* NIH198. Our results showed that fluconazole was synergistic with sodium butyrate or with trichostatin A for the *hda1*Δ/*hos2*Δ double mutant strain. Sulfamethoxazole was synergistic with sodium butyrate or with hydralazine also for *hda1*Δ/*hos2*Δ. These results clearly indicated a link between histone deacetylase (HDAC) impairment and drug sensitivity.

Due to limitations for the treatment of *C. neoformans* infections, alternative treatments such as photodynamic therapy, involving aluminium phthalocyanine chloride (NE-AlPcCl) nanoemulsion in combination with the aforementioned drugs, could help in circumventing this problem. We observed, also in Chapter I, that amphotericin B, fluconazole, sodium butyrate (HDACi) or hydralazine (DNMTi) potentiated the antimicrobial activity of photodynamic therapy against all the strains.

The microbial increasing drug resistance stimulates the exploration of new antifungal compounds from different sources, including metabolites from actinobacteria and other fungi. Our research group has a long tradition of scientific cooperation with India so, in Chapter II, in collaboration with the Department of Microbiology of the Maharshi Dayanand University (Rohtak, India), we purified and identified four actinobacteria isolates (Ha1, Pp1, UzK and UzM) from the rhizosphere soil of Indian traditional medicine plants. The effect of the crude extract of these isolates on the growth of the aforementioned *Cryptococcus* strains was assessed. Fluconazole-resistant strains demonstrated higher susceptibility to the crude extract of Pp1 (*S. griseoruber*) in comparison to the reference strain H99. *C. gattii* NIH198 was sensitive to the crude extract of UzK and UzM (*S. aureus*). HDAC genes deletion strains were more susceptible to the crude extract of all the *Streptomyces* isolates.

Since filamentous fungi also pose serious treats to human health, in Chapter III, we evaluated the antifungal activity of the *Streptomyces* isolates from India against filamentous fungi we isolated from the iron ore plateau of The Carajás National Forest, Pará, Brazil (*Fusarium striatum*, *F. solani*, *Mucor fragilis* and *Penicillium citrinum*), against *Chaetomium madrasense*, isolated as a laboratory contaminant in India, and against *Aspergillus terreus*, *A. flavus* and *A.* fumigatus. Overall, in co-culture experiments, the *Streptomyces* isolates inhibited the fungi mycelial growth by 20 to 90%, depending on the species.

Our results presented a clear link between HDAC impairment, drug susceptibility and the sensitivity to photodynamic therapy in *Cryptococcus*. Our data, on yeasts and filamentous fungi, stimulate the design of alternative antifungal therapies by combining epigenetic modulators, photodynamic therapy, and actinobacteria-derived metabolites.

Keyword: *Cryptococcus*, antifungal drugs, epigenetic modulators, photodynamic therapy, synergism, actinobacteria antifungal activity.

RESUMO

Cryptococcus spp. são patógenos fúngicos oportunistas distribuídos globalmente que afetam principalmente indivíduos imunocomprometidos. As opções terapêuticas são limitadas e incluem principalmente anfotericina B ou fluconazol, isoladamente ou em combinação. O uso extensivo de antifúngicos vem ocasionando a seleção de patógenos resistentes, o que representa séria ameaça à saúde pública global. Genes de histona desacetilase estão envolvidos na virulência de *Cryptococcus neoformans* e na patogenicidade e resistência a azóis em *Candida albicans*. Nesse contexto, no Capítulo I desse trabalho, avaliamos a atividade dos antifúngicos anfotericina B, fluconazol, sulfametoxazol, dos inibidores de histona desacetilase (HDACi) butirato de sódio ou tricostatina A e dos inibidores de DNA metiltransferase (DNMTi) hidralazina ou 5-aza-2 ′-desoxicitidina, isoladamente e em combinações, sobre o crescimento das linhagens de *C. neoformans* H99, T1 e 89-610 (resistentes a fluconazol) e mutantes nulos para genes de histona desacetilase (*hda1*Δ, *hos2*Δ, *hda1*Δ / *hos2*Δ e *hda1*Δ + HDA1), assim como de *C. gattii* NIH198. Nossos resultados demonstraram que fluconazol foi sinérgico com butirato de sódio ou com tricostatina A para a linhagem mutante duplo *hda1*Δ / *hos2*Δ. Sulfametoxazol foi sinérgico com butirato de sódio ou com hidralazina também para *hda1*Δ / *hos2*Δ. Tais resultados indicam claramente uma ligação entre o comprometimento da atividade de histona desacetilase (HDAC) e a sensibilidade de *Cryptococcus* a antifúngicos.

Devido às limitações para o controle da infecção por *Cryptococcus*, tratamentos alternativos, como a terapia fotodinâmica envolvendo nanoemulsão de cloreto de ftalocianina de alumínio (NE-AlPcCl), em combinação com as drogas acima mencionadas, podem contribuir com a otimização dos esquemas terapêuticos. Observamos, também no Capítulo I, que anfotericina B, fluconazol, butirato de sódio (HDACi) ou hidralazina (DNMTi) potencializaram a atividade antimicrobiana da terapia fotodinâmica sobre todas as linhagens.

O aumento da resistência microbiana a drogas estimula a pesquisa de novos compostos antifúngicos de diferentes fontes, incluindo metabólitos de actinobactérias. Como nosso grupo de pesquisa tem longa tradição de cooperação científica com a Índia, no Capítulo II, em colaboração com o Departamento de Microbiologia da Maharshi Dayanand University, (Rohtak, Índia) purificamos e identificamos quatro isolados de actinobactéria (Ha1, Pp1, UzK e UzM) a partir da rizosfera de plantas da Medicina Tradicional Indiana. Os extratos brutos derivados desses isolados foram testados quanto à inibição do crescimento das linhagens de *Cryptococcus* mencionadas acima. As linhagens resistentes a fluconazol demonstraram maior suscetibilidade ao extrato bruto de Pp1 (*S. griseoruber*), em comparação à linhagem de referência H99. *C. gattii* NIH198 apresentou maior susceptibilidade ao extrato bruto de UzM

(*S. aureus*). As linhagens mutantes para HDAC, quando comparadas a H99, mostraram-se mais sensíveis aos extratos brutos de todos os isolados de *Streptomyces*.

Como fungos filamentosos também podem representar séria ameaça à saúde humana, no Capítulo III desse trabalho avaliamos, por co-cultura, a atividade antifúngica dos isolados de *Streptomyces* indianos contra fungos filamentosos que isolamos da área ferrífica da Floresta Nacional de Carajás, Pará, Brasil (*Fusarium striatum*, *F. solani*, *Mucor fragilis* e *Penicillium citrinum*), contra *Chaetomium madrasense*, isolado como contaminante laboratorial na Índia e contra linhagens laboratoriais de *Aspergillus terreus*, *A. flavus* e *A. fumigatus*. Todos os isolados de *Streptomyces* inibiram o crescimento micelial dos fungos testados de 20 a 90%, dependendo da espécie.

Nossos resultados apresentaram uma clara ligação entre o comprometimento da atividade de HDAC e a suscetibilidade a drogas e à terapia fotodinâmica em *Cryptococcus*. Nossos dados com essas leveduras e com fungos filamentosos estimulam o desenho de abordagens terapêuticas alternativas que combinem antifúngicos tradicionais, moduladores epigenéticos, terapia fotodinâmica e metabólitos derivados de actinobactérias.

Palavras-chave: *Cryptococcus*, drogas antifúngicas, moduladores epigenéticos, terapia fotodinâmica, sinergismo, atividade antifúngica de actinobactérias.

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
AlPcCl	Aluminum phthalocyanine chloride
AMB	Amphotericin B
BBB	Blood brain barrier
CNS	Central nervous system
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methytransferase
DNMTi	DNA methytransferase inhibitors
FLU	Fluconazole
HAT	Histone acetyletransferses
HDAC	Histone deacetylases
HDACi	Histone deacetylase inhibitors
HIV	Human immunodeficiency virus
HLZ	Hydralazine
MICs	Minimum inhibitory concentrations
mya	Million years ago
NaBut	Sodium butyrate
NAD^+	Nicotinamide adenine dinucleotide
PBS	Phosphate buffered saline
PDT	Photodynamic therapy
pH	Hydrogen potential
PS	Photosensitizers
ROS	Reactive oxygen species
rpm	Rotation per minute
SMX	Sulfamethoxazole
TSA	Trichostatin A
5-AZA	5-aza-2′-cytidine
5-AZAdc	5-aza-2′-deoxycytidine

SUMMARY

CHAPTER I

The role of *Cryptococcus neoformans* histone deacetylase genes in the response to antifungal drugs, epigenetic modulators and to photodynamic therapy mediated by an aluminium phthalocyanine chloride nanoemulsion *in vitro*

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1.1. INTRODUCTION

Fungi are eukaryotic organisms commonly found in different environments; some species are considered commensals, present in the animal microbiota (CARMONA; LIMPER, 2017; PATERSON; OH; UNDERHILL, 2018). Fungi have been highlighted as human pathogens, mainly for certain groups of patients, significantly increasing the morbidity and mortality (PFALLER; DIEKEMA, 2007; LASS-FLÖRL, 2009; PILMIS et al., 2016). Patients admitted to intensive care units, infected with HIV and those undergoing cancer or immunomodulatory treatments represent groups at risk for fungal infections (reviewed by PAPPAS, 2010). These infections include a wide range of diseases that affect different tissues and organs (reviewed by NAMI et al., 2019). Systemic infections represent a serious threat.

Although many fungal genera can cause such infections, species of the genus *Cryptococcus* are commonly associated with diseases in humans. These species are associated with severe opportunistic infections in the world and can cause cryptococcosis (KWON-CHUNG et al., 2015; RAJASINGHAM et al., 2017).

1.2 *Cryptococcus*

Cryptococcus spp. are basidiomycetes which live as single cells or form biofilms. SANFELICE (1894) first isolated *Cryptococcus* cells from fermented peach juice samples. After that, different species have been isolated around the globe. Most species are isolated from plants (LI et al., 1995; JAGER; WEHNER; KORSTEN, 2001), bird faeces (MANCIANTI; NARDONI; CECCHERELLI, 2001), from lakes (ROSA et al., 1995) and marine environments (KUTTY; PHILIP, 2008). The *Cryptococcus* genus was described as a dominant fungal group in soils (VALINSKY et al., 2002). About 37 *Cryptococcus* species have been described; most

of them are non-pathogenic. *C. neoformans* and *C. gattii* are the major pathogens to humans and animals, while *C. alurantii* and *C. albidus* can rarely cause diseases.

Different typing methods were employed to assess genetic diversity in *Cryptococcus*. These methods include 1) AFLP analysis (COGLIATI, 2013), 2) PCR fingerprinting (CHEN; MEYER; SORRELL, 2014) , 3) *URA5* sequencing, 4) *PLB1* gene (TRILLES et al., 2014) and 5) MLST genotyping (BEALE et al., 2015).

1.3 Cryptococcosis

Cryptococcosis is an opportunistic disease that mainly affects immunocompromised individuals, such as HIV/AIDS patients, patients undergoing chemotherapy or transplant recipients treated with immunosuppressants (KERKERING; DUMA; SHADOMY, 1981), This disease is associated with high treatment costs and high lethality. Initially, the disease was attributed uniquely to *C. neoformans*. However, more refined molecular methods have led to the identification of a different strain, *Cryptococcus neoformans* var. *gattii*, which was later classified as the distinct species *C. gattii* (serotypes B and C) (KWON-CHUNG et al., 2015).

Cryptococcus infection occurs through the inhalation of infectious particles, such as small, desiccated yeasts or basidiospores present in the environment. After reaching the pulmonary alveoli, the fungus can proliferate (Figure 1). In the lungs, pulmonary macrophages are the main phagocytic cells and, therefore, the first cells of the immune system to defend the host against this pathogen. However, *C. neoformans* is an facultative intracellular pathogen, capable of surviving and replicating intracellularly (DE LEON-RODRIGUEZ et al., 2018; NELSON; HAWKINS; WOZNIAK, 2020). The ability of this pathogen to survive inside macrophages probably contributes to chronic and latent infections (HARDISON et al., 2010;DAVIS et al., 2015; DRAGOTAKES; FU; CASADEVALL, 2019). The fungus can spread through the bloodstream and almost any organ can be infected, such as joints, skin, eyes,

urinary tract and liver (VECCHIARELLI et al., 1996);ESHER; ZARAGOZA; ALSPAUGH, 2018 . Skin infections generally reflect widespread disease, although rare cases of primary cutaneous cryptococcosis were also described (CHRISTIANSON; ENGBERT; ANDES, 2003; HAYASHIDA et al., 2017; JARAŠŪNIENĖ et al., 2020).

C. neoformans can cross the blood-brain barrier (BBB) through transcellular (penetration through brain microsvascular endothelial cells), paracellular (penetration after the biochemical disruption of endothelial cells) and Trojan Horse (immune cells carries the pathogens through the BBB) mechanisms, and spread into the central nervous system (CNS), causing cryptococcal meningitis, the most severe clinical condition of the disease (Reviewed by ESHER; ZARAGOZA; ALSPAUGH, 2018; Figure 1).

AARON and colleagues (2018) described five signal transduction pathways in human brain endothelial cells: EPH-ephrinA1 receptor (EphA2), axonal guidance (related to EPH signalling), RhoGDI, CXCR4 and IL-8 (CXCL8) signalling pathways. These pathways are the main target for *Cryptococcus* to breach the endothelium. These authors reported that *C. neoformans* bind to brain endothelium cells and induce EphA2 phosphorylation through CD44. The phosphorylation of EphA2 promotes the GTPase-dependent signalling and the recognition of the actin cytoskeleton, which leads to the internalisation of *C. neoformans* and crossing of the BBB. *C. neoformans* induced the expression of CXCR12, which promotes the CXCL4 mediated signalling, supporting the Trojan Horse mechanism via infected phagocytes.

LAHIRI and collaborators (2019) studied the invasion and survival of *C. neoformans*/*gattii* in the CNS. Clinical isolates of *Cryptococcus* presented a higher rate of invasion and survival in human brain microvascular endothelial cell (HBMEC) lines when compared to environmental samples. *C. neoformans* displayed a higher rate of invasion and survival in HBMEC than *C. gattii*.

LEE and colleagues (2020) studied the transcriptional profile of transcription factors, kinases and virulence related genes during *Cryptococcus* infections. The authors demonstrated that *Cryptococcus* requires four kinases (Pkh201, Alk1, Irk2 and Vrk1) and five transcription factors (Ada2, Hap2, Sre1, Hob1, and Pdr802) to cross the BBB and to survive in the brain parenchyma cells. They indicated that biological processes such as lipid-mediated regulation, cell cycle regulation, chromatin dynamics, and haeme-mediated respiration control are crucial in this process.

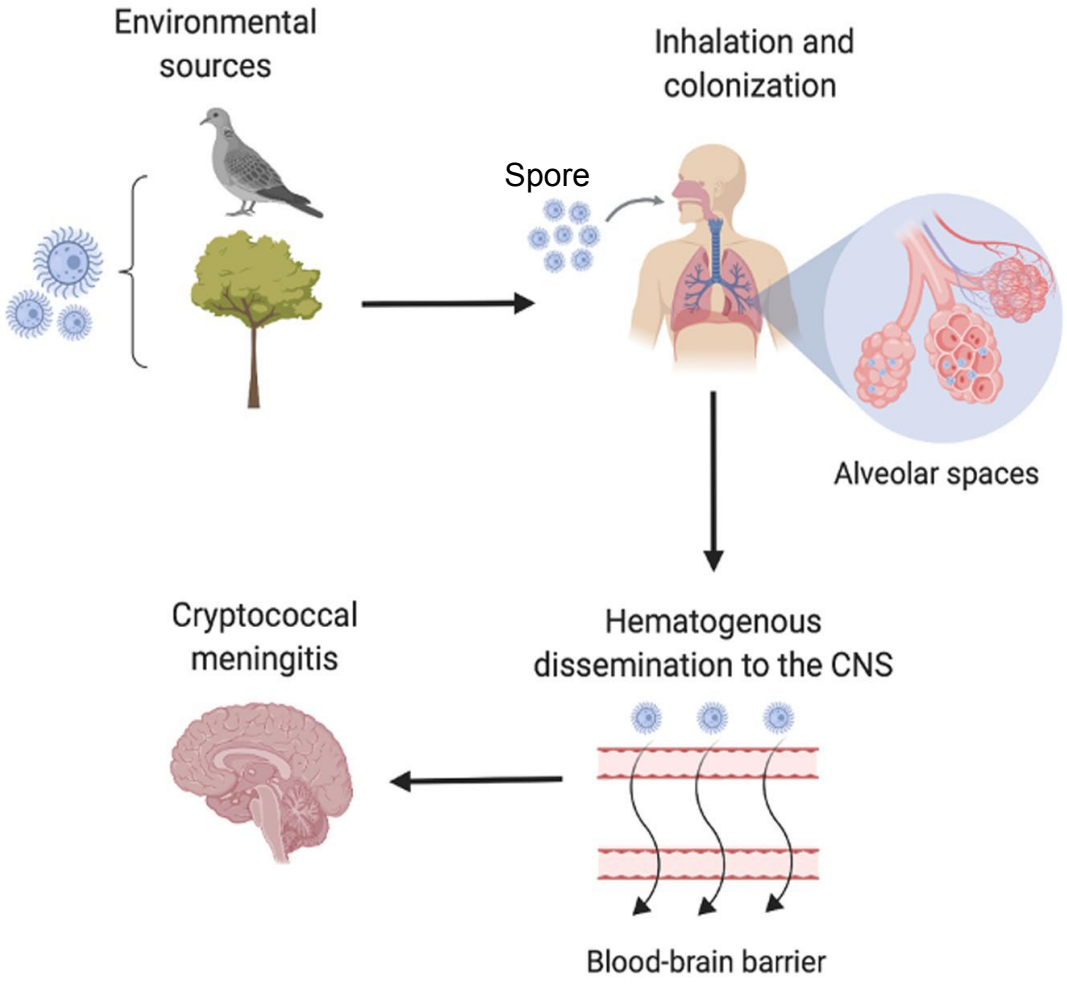


Figure 1. *Cryptococcus* infection cycle. *Cryptococcus* in nature can be found in pigeon droppings or in plant debris. Infection occurs by inhaling infectious particles, such as yeasts or spores. If the infection is not eradicated by the immune system, the fungus invades the pulmonary alveoli, where it can proliferate. The disease can spread and reach the central nervous system, progressing to cryptococcal meningitis. Adapted from (BERMAS; GEDDES-MCALISTER, 2020).

In immunocompetent hosts, most primary infections are believed to be asymptomatic. The study by CHEN et al. (1999) demonstrated the presence of antibodies reactive to 20467 *C. neoformans* proteins in most healthy individuals. The development of the disease will depend on the response of the host immune system. It can become latent with the pathogen contained in granulomas, being reactivated later, or it evolves to the acute form (PERFECT; CASADEVALL, 2002) which can be fatal if untreated.

An exact assessment of a cryptococcal infection burden is important for prevention and regulatory strategies. PARK and colleagues (2009) estimated, 957,900 cases of cryptococcal infection and 624,725 of deaths among HIV infected persons during the pre-ART (antiretroviral therapy) era. Approximately 504,000 deaths were reported in Sub-Saharan Africa, the most affected region of world (COGLIATI, 2013)

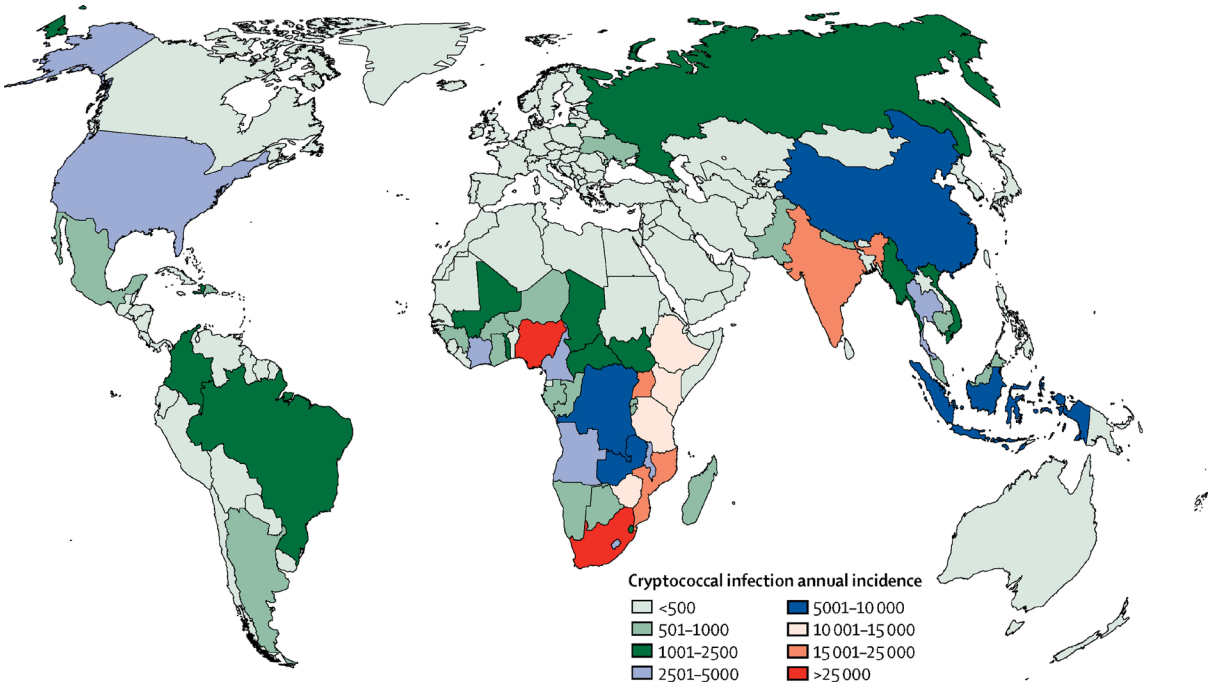


Figure 2: Annual incidence of cryptococcal infection by country in 2014. Adapted from RAJASINGHAM et al., (2017).

It is estimated that more than 223,000 cases of cryptococcal meningitis occur each year worldwide, of which about 70% occur in sub-Saharan Africa (Figure 2). In 2014, this disease resulted in 180,100 deaths, and 75% of them occurred in sub-Saharan Africa.

1.4 Antifungal drugs and prevalence of drug-resistant *Cryptococcus isolates*

There are numerus class of antibiotics available to treat bacterial infections, but antifungals are mainly restricted to three classes: polyenes (amphotericin B), azoles (fluconazole, itraconazole, voriconazole, posaconazole) and echinocandins. *Cryptococcus* is intrinsically resistant to echinocandins due to the interaction of Cdc50 (lipid flippase) and Crm1 (mechanosensitive calcium channel proteins), which regulate the intracellular calcium homeostasis and echinocandin resistance via the calcium/calcineurin signalling. Only polyenes and azoles represent treatment options (CAO et al., 2019; reviewed by NICOLA et al., 2019; STONE et al., 2019).

For more than a half century, amphotericin B (AMB) has been the primary treatment option for cryptococcal infections. The extensive use of deoxycholate AMB (d-AMB) causes nephrotoxicity. To reduce this toxicity, liposome bilayer-coated amphotericin B (l-AMB) was developed. This formulation presented a lower toxicity (Jarvis et al., 2019). FALCI and collaborators (2015) assessed the frequency of nephrotoxicity with d-AmB and l-AmB in 431 patients admitted at Santa Casa de Misericordia de Porto Alegre, Southern Brazil between 2003 and 2012. Nephrotoxicity was more frequent upon treatment with d-AMB (11.5%) in comparison to l-AMB (2.4%). Treatment with d-AMB, for minimizing the risk of opportunistic fungal infections, caused renal failure in 36.4 % of the patients (n=110) with haematological malignancies (GURSOY et al., 2021).

A new nanoparticle-based encochleated amphotericin B (C-AMB) formulation was developed. Cochleate is composed of negatively charged lipids and divalent cations such as

calcium. In this formulation, encapsulation prevents the gastrointestinal degradation of AMB, enabling the oral administration, and it was effective in delivering the drug to the central nervous systems (CNS). CAmB is now under clinical trials phase I and II for cryptococcal meningitis (Reviewed by AIGNER; LASS-FLÖRL, 2020).

Fluconazole (FLU) was approved to human treatment in early 1990s. FLU is advantageous in comparison to AMB due to the bioavailability (100%), tolerance to gastrointestinal pH and CNS permeability. On the other hand, FLU is fungistatic, so it is less effective than the fungicide AMB. In this view, FLU monotherapy is not recommended, since it requires an extended and high dosage treatment. FLU in combination with AMB is more efficient in treating cryptococcosis. Clinical trials also demonstrated that FLU is more effective in combination with AMB (MOLLOY et al., 2018; XU et al., 2019).

Cryptococcus resistance to AMB, FLU or both is increasing and is associated with therapeutic failures. BONGOMIN and colleagues (2018) evaluated the prevalence of FLU resistance in *Cryptococcus* from 1988 to 2017 using the EMBASE and MEDLINE databases to track all the articles presenting the FLU susceptibility data of clinical isolates of *Cryptococcus*. Overall, ten percent of the 4995 *Cryptococcus* isolates derived from 3210 patients were FLU resistant. The authors also included 248 isolates from relapsed episodes of cryptococcosis; these isolates presented a higher rate of resistance (24.1%). Thirty eight percent of the studies employed the MICs breakpoints of $\geq$64 μg mL^{-1} to define FLU resistance, 21% considered $\geq$32 μg mL^{-1}, 38% $\geq$16 μg mL^{-1} and 3% $\leq$20 μg mL^{-1}.

PHARKJAKSU and collaborators (2020) determined the *in vitro* antifungal susceptibility patterns of 233 isolates from the *C. neoformans*/*gattii* Species Complexes, collected at National Institutes of Health centres, USA. *C. neoformans* species complex comprised 89.7% of the isolates and *C. gattii* 10.3% isolates. *C. gattii* species complex

demonstrated higher MIC values for FLU in comparison to the *C. neoformans* species complex. *C. neoformans*/VNI strains displayed higher MICs values to fluconazole than *C. neoformans*/VNII, *C. neoformans*/VNII, while *C. deneoformans*/VNIV was more resistant to AMB and FLU. The 167 clinical *C. neoformans* species complex isolates displayed different ranges of MICs for AMB: 50.9% were inhibited by 1 μg mL^{-1}; 14.4% by 2 μg mL^{-1}, and 1.8% by 4 μg mL^{-1}.

There are numerous studies available on the mechanisms of drug resistance in *Cryptococcus*. BOSCO-BORGEAT and colleagues (2016) detected the presence of the G1855A nucleotide point mutation in the ERG11 gene, encoding lanosterol 14-α-demethylase in clinical isolates with high FLU MIC values (>16 μg mL^{-1}). This point mutation resulted into the G484S amino acid substitution, located in the active site of the enzyme. The authors proposed that this amino acid substitution is involved in FLU resistance in *Cryptococcus*.

Phenotypic and genotypic studies were performed for 20 clinical *Cryptococcus* isolates obtained from patients receiving FLU monotherapy or in combination with 5-fluorocyotsine (5FC), 19 isolates belong to *C. neoformans* (VNI) and one to *C. gattii* (VGI). *Cryptococcus* isolates obtained from patient receiving FLU monotherapy displayed greater diversity in the population of resistant cells (heteroresistance) than those receiving the FLU/5FC combination therapy. Combination therapy suppresses the amplification of heteroresistant cells. Aneuploidy of chromosome 1 (Chr1) was detected as the dominant feature in heteroresistant cells, as well as in the relapsed isolates (STONE et al., 2019).

Whole genome sequencing of 16 independent isolates of *Cryptococcus* resistant to 5-fluorocytosine revealed a mutation in the *UXS1* gene, encoding an enzyme that synthesizes UDP-xylose from UDP-glucuronic acid, required for the capsule biogenesis. *UXS1* mutation resulted in the accumulation of UDP-glucuronic acid, in the alteration of nucleotide

metabolism, and in the suppression of the toxicity of 5-fluorocytosine and its derivatives (JANBON et al., 2014).

GUSA and colleagues (2020) described an enhanced rate of transposon mutagenesis when *Cryptococcus* was incubated at 37° C in comparison to 30° C, *in vitro,* upon the inactivation of the RNA interference (RNAi) pathway. These two temperature conditions mimic the temperature shift of pathogenic microorganism from the environment to mammal hosts. The authors also demonstrated that transposon mutagenesis drives the development of 5-fluoroorotic acid (5FOA) resistance in a murine model of infection.

Multiple studies reported the role of epigenetic changes in the phenotype plasticity associated with the ability of pathogenic fungi to stablish infection in host organisms (Reviewed by SLEPECKY; STARMER, 2009; ZAFAR et al., 2019).

1.5 Epigenetic modifications

Conrad Waddington introduced the term “epigenetics” in 1942 to describe “the interactions of genes with their environment that bring the phenotype into being”. Nowadays, it refers to inherited modifications in gene function that are not related to alterations in the DNA sequence. Epigenetic changes involve DNA methylation, histone posttranslational modifications, histone variants, non-coding RNAs and chromatin remodelling complexes. DNA methylation and the histone posttranslational modifications are the most studied aspects. These two alter the DNA accessibility and chromatin structure, thereby modifying the gene expression patterns (JABLONKA; LAMB, 2002; HOLLIDAY, 2006).

1.6 DNA methylation

DNA methylation is catalysed by enzymes known as DNA methyltranferases (DNMTs). This reaction occurs mainly in the 5th position (C5) of cytosine nucleotides that are

found next to guanine nucleotide in the DNA sequence (CpG dinucleotides), although other methylation contexts can also occur such as CHG, CHH (H is A, T or C) (MOORE; LE; FAN, 2013; SKVORTSOVA; STIRZAKER; TABERLAY, 2019).

The study of the genome-wide DNA methylation pattern in the fungus *Heterobasidion parviporum* (ZENG et al., 2019) revealed that the relative proportion of methyl cytosine sites are 45-72% for CHH, 30-36% for CpG and 17-18% for CGH. Transposable elements were heavily methylated in both CpG (>90%) and non-CpG (>20%) contexts. The methylation patterns of all contexts were also reported for exons, introns and for 1.5 kb up- and 1.0 kb downstream of genes. In the CpG context methylation level of genes were lower than for the flanking regions.

There are mainly two mechanisms by which cytosine methylation may affect transcription. First, the methyl group itself may obstruct the recognition and/or binding of transcription factors to the gene regulatory elements. Second, methylated DNA may be bound by methyl-CpG-binding domain proteins (MBDP), which then recruit repressor proteins, like histone deacetylases and chromatin remodelling factors, leading to a compact and inactive chromatin status (Reviewed by SKVORTSOVA; STIRZAKER; TABERLAY, 2019).

DNA methylation activity can be classified into two types: *de novo* and maintenance. In *de novo*, cytosine methylation occurred at unmethylated DNA, while in the maintenance type, unmethylated cytosine residues of hemi-methylated DNA segments are methylated after DNA replication. So far, four DNMTs have been identified in mammals: DNMT1 for the maintenance of methyltransferase and Dnmt3A, Dnmt3B and Dnmt3C for the *de novo* activity (Reviewed by UNOKI, 2019).

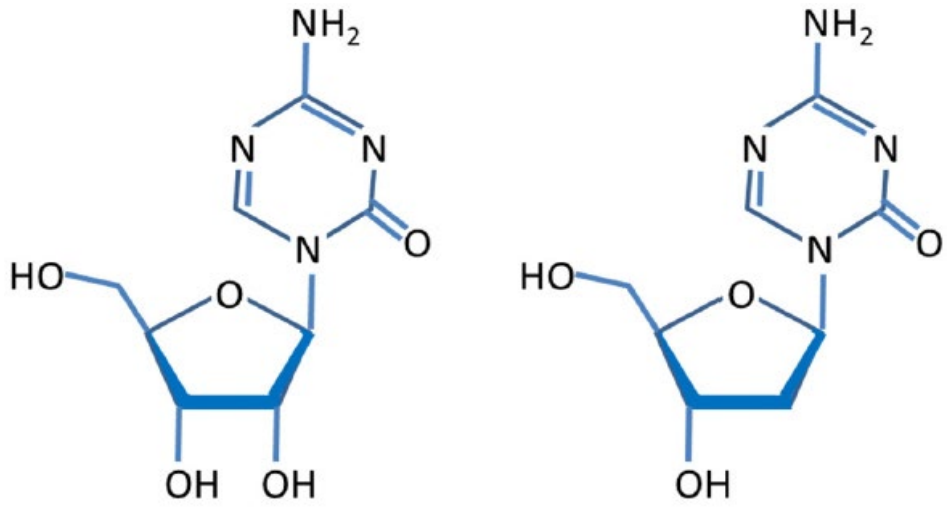
In *C. neoformans* CpG methylation requires Dnmt5, a maintenance type enzyme (CATANIA et al., 2020). By analysing the genome of species close to *C. neoformans*

(*Kwoniella mangroviensis*, *K. bestiolae*, and *K. pini*), the authors revealed that the DnmtX *de novo* methytransferase was lost in *C. neoformans* between 150 and 50 mya. In this view, no efficient *de novo* methylation was described for this fungus. On the other hand, the authors characterized a maintenance DNMT (Dnmt5) which is responsible for efficient propagating DNA methylation through mitosis and meiosis, by employing hemi methylated DNA as substrate, particularly at transposons, and at the centromeres and telomeres vicinity. These data point out to peculiar features of the epigenetic landscape of *C. neoformans*.

Studies demonstrated that the methylation of adenine on the 6th position (m^6A) is also highly conserved across eukaryotes. In comparison to cytosine methylation, m^6A abundance is lower, ranging from 0.000006% to 0.8%. It regulates transcription activation and silencing, chromatin remodelling and stress response (MONDO et al., 2017; XIAO et al., 2018). Mondo and collaborators (2017) analysed the genome of 16 phylogenetically diverse fungi using single-molecule real-time techniques for the presence of m^6A. Distribution of m^6A was inversely correlated with cytosine methylation. Authors also suggested that m^6A is symmetrically methylated and acts as a heritable DNA modification in fungi. There is no information on m^6A in *Cryptococcus*.

1.7 DNA methyltransferase inhibitors (DNMTi)

There are two class of DNMTi: 1) a nucleotide analogue binds to DNA to form a covalent complex that promotes the degradation of DNMT and 2) a non-nucleotide analogue DNMTi which binds directly to the methylated region of the DNMT. 5-azacitidine (5-Aza) and 5-aza-2′deoxycytidine (5-AzadC) are representatives of the nucleotide analogue class (Figure 3) (DAN et al., 2019).



5-azacitidine 5-aza-2′deoxycytidine

Figure 3: DNMT inhibitors: Representative structure of the 5-azacitidine and 5-aza-2′deoxycytidine. Adapted from SCHWARZENBACH; GAHAN, (2019)

In *C. albicans*, 5′ -azacytidine synchronized and accelerated the germ tube formation during the yeast-mycelium transition; this suggested that DNA demethylation is important for the regulation of the dimorphic transition associated with virulence (PANCALDI et al., 1988). In *A. nidulans*, 5-azacytidine induced the abnormal fluffy phenotype, and delayed conidia formation (TAMAME; ANTEQUERA; SANTOS, 1988). A lower risk of invasive fungal infections was reported when acute myeloid leukaemia and myelodysplastic syndromes patients were treated with 5-azacytidine (POMARES et al., 2016). Both 5-Aza and 5-AzadC are approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of acute myeloid leukaemia (AML), chronic myelomonocytic leukaemia (CMML), and myelodysplastic syndromes (MDS) (KAMINSKAS et al., 2005; STEENSMA et al., 2009; ERDMANN et al., 2015).

The exposure to 5-AZA reduced aflatoxin production and also changed the morphology of *Aspergillus flavus* (YANG et al., 2015). Later, SONG and collaborators (2020) demonstrated that 5-AZA induced severe defects in the aflatoxin biosynthesis and fungal development in this

fungus. A non-aflatoxigenic A. *flavus* strain was obtained and it produced less fatty acid-derived volatiles, important precursors for the aflatoxin biosynthesis.

Hydralazine (HLZ), a non-nucleotide analogue DNMTi has been widely used to treat hypertension during pregnancy as well as heart failure. Later, it has been shown to act as a DNA methylation inhibitor by reducing the expression of the DNA methyltransferases (Deng et al., 2003; Arce et al., 2006). It is under evaluation under clinical trials for cancer therapy alone and/or in combination with histone deacetylase inhibitors HDACi (CANDELARIA et al., 2017; SCHCOLNIK-CABRERA; DOMÍNGUEZ-GÓMEZ; DUEÑAS-GONZÁLEZ, 2018; ANDRADE et al., 2019). The understanding of the mechanism of action as a DNA demethylating agent is very limited. There are no reports on the exposure of pathogenic fungi to HLZ.

1.8 Histone modifications

Histone post-translational modifications, including methylation, phosphorylation, acetylation, ubiquitylation and sumoylation, have been indicated as a pivotal component of the epigenetic landscape. Histone acetylation is highly dynamic and regulated by enzymes called histone acetyltransferases (HATs) and histone deacetyl transferase (HDACs). HATs catalyse the transfer of the acetyl group to the ε-amino group of lysine side chain by utilizing acetyl-coA as a cofactor. The acetyl group reduces the positive charge of lysine residue thus leading to the reduced affinity between the histone and the DNA. DNA then opens up and become accessible to the transcription machinery. HDACs catalyse the removal of acetyl group from lysine residue and repress the transcription (BANNISTER; KOUZARIDES, 2011).

1.9 Histone deacetylases (HDACs)

The HDACs in fungi are grouped in three different classes. Classes I and II englobe the Rpd3 and Hda1 homologs from *S. cerevisiae*, respectively. Class III comprises HDACs which

require NAD^+ as a cofactor.They are closely related to *S. cerevisiae* Sir2, and are collectively called sirtuins (BRACHMANN et al., 1995; FRYE, 1999; JACKSON; DENU, 2002).

Considering pathogenic fungi, histone acetylation/deacetylation role in virulence is well described for *C. albicans*. HDACs were associated with the regulation of virulence traits, such as antifungal drugs resistance, the transition between the white-opaque phenotypes and the adhesion to lung cells (KLAR; SRIKANTHA; SOLL, 2001; SMITH; EDLIND, 2002). ZACCHI (2006) demonstrated that HDACs Hos2 and Hda1 play an important role in the regulation of the morphological transition; the first being a repressor and the second an inducer of filamentation. The Set3 / Hos2 deacetylase complex also regulates morphogenesis and virulence in *C. albicans* (HNISZ et al., 2010). The HDACs Hda1 and Rpd3 of *S. cerevisiae* and *C. albicans* are important to antifungal drugs resistance by regulating the Hsp90 chaperone acetylation (ROBBINS; LEACH; COWEN, 2012).

In *C. neoformans,* seven HDAC genes were identified: *hos1*, *hos2*, *rpd3*, *clr61* and *clr62* for Class I, and *hda1* and *hos3* for Class II HDAC (BRANDÃO et al., 2018). By gene deletion, the authors reported that, amongst the seven HDAC genes identified in the *C. neoformans* genome, *hda1* and *hos2* are the most prominent for fungal virulence attributes both *in vitro* as in animal models of infection. They also demonstrated that Class I and II HDAC genes play distinct and overlapping roles and act as key regulators of *C. neoformans* virulence. There are no reports on a possible role of HDAC genes in drug resistance in *Cryptococcus.*

1.10 Histone deacetylase inhibitors (HDACi)

Histone deacetylase inhibitors (HDACi) are studied as drugs for the treatment of cancer and of HIV-infected culture cells, arousing great interest in the pharmaceutical industry (WEST; SMYTH; JOHNSTONE, 2014). The FDA has approved a few HDACi such as

vorinostat, romidepsin and belinostat for the treatment of cutaneous/ peripheral T-cell lymphomas (MIRANDA FURTADO et al., 2019).

Several HDACi have been isolated from natural sources or synthesized in laboratories. Sodium butyrate (NaBut) (Figure 4) is a short-chain fatty acid, belonging to the group of aliphatic acids, produced by the fermentation of anaerobic bacteria and which inhibits class I and IIa HDACs (SAWAN et al., 2008; HALSALL et al., 2012; SIMON-O'BRIEN et al., 2015).

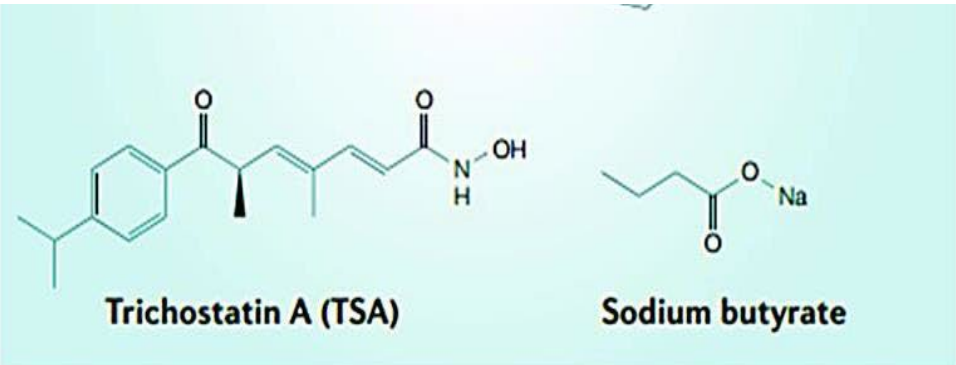


Figure 4: HDAC inhibitors: Representative structure of the main HDACi classes: Trichostatin A and Sodium butyrate. Adapted from MINUCCI; PELICCI, (2006).

Trichostatin A (TSA) (Figure 4) is a hydroxamate derivative initially characterized as a product of the metabolism of *Streptomyces hygroscopicus* (TSUJI et al., 1976). It was the first hydroxamic acid identified as a specific HDAC inhibitor, affecting all classes of HDACs (HU et al., 2003; MARKS, 2010). The mechanism of action for this compound remains unclear.

NaBut inhibited the capsule formation in *Cryptococcus* and also inhibited the yeast growth and filamentation in *C. albicans*. Antifungal activity of FLU increased upon exposure to NaBut. It also significantly increased the fungicidal activity of macrophages (NGUYEN et al., 2011).

BRANDÃO and collaborators (2015) studied the effect of two distinct HDACi (NaBut and TSA) on *C. neoformans* virulence phenotypes, *in vitro*. Both drugs affected the growth of

C. neoformans at 37°C and also reduced the capsule expansion, and impaired the mating hyphae development. Only NaBut inhibited the phospholipase activity and affected the cell cycle progression, as it increased the cell population arrested at the G2/M phase of the cell cycle.

NaBut completely inhibited the planktonic cells of *Trichosporon asahii* and *T. inkin* at 60 or 120 mM. The drug reduced the fungi metabolic activity up to 63% and also reduced biomass up to 81% (CORDEIRO et al., 2019).

Recently, LI and colleagues (2021) designed and synthesised twelve carboline compounds as a new fungal HDACi. Especially, compound 12 showed synergistic effect with FLU against FLU-resistant *C. albicans,* by blocking the morphological transition and by preventing biofilm formation. This compound also reversed the drug resistance by downregulating the *ERG11* and *CDR1* genes expression.

Some studies have demonstrated the ability of HDACi to interact with phototherapy, increasing the cytotoxic effect of photodynamic therapy (PDT) on human cancer cells (HALABURKOVÁ et al., 2017; SUNG; VERVERIS; KARAGIANNIS, 2014; YE et al., 2014). There are limited studies on the response of cancer cells to the combined exposure of HDACi or DNMTi and photodynamic therapy.

1.11 Photodynamic therapy (PDT)

PDT is a promising therapeutic approach based on oxidative stress generated by type I and type II photoreactions, triggered by the photoactivation of a photosensitizing (PS) agent in a medium containing molecular oxygen.

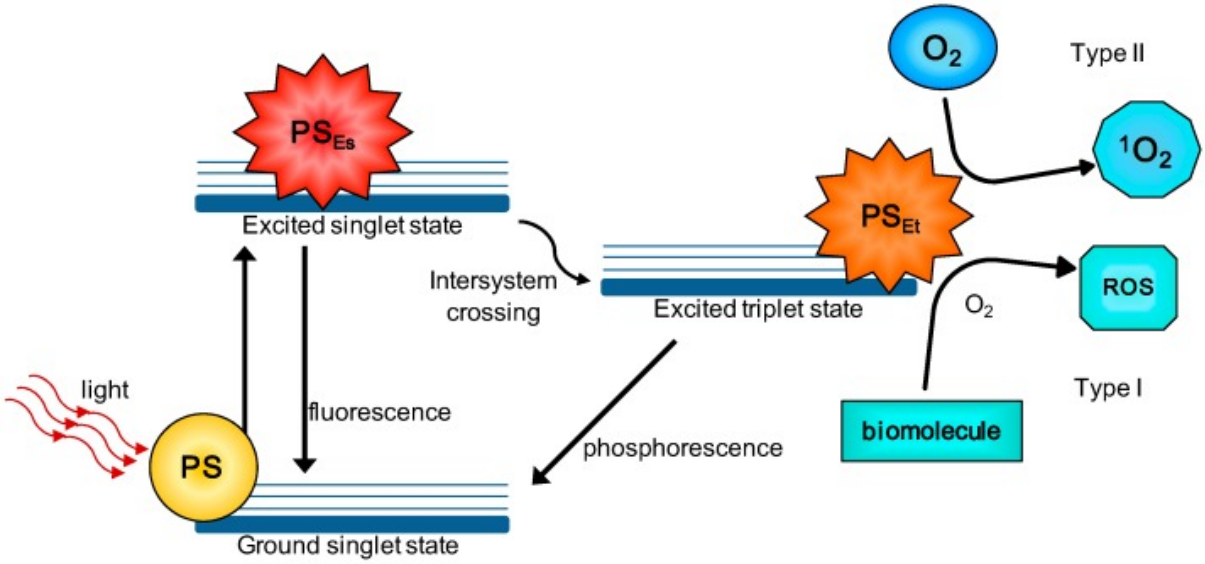


Figure 3: Schematic representation of the mechanism of action of photodynamic therapy (PDT). The PS reached excited triplet state by following excited singlet state. It follows two pathways: type I, react with neighbouring substrates by transferring electron to generate radicals, which further reacts with molecular oxygen to generate ROS; or, Type II reaction, triplet PS directly react with molecular oxygen, generates singlet oxygen. PS denotes photosensitizer; PSEs, excited PS (singlet state); PSEt, excited PS (triplet state); ROS denotes reactive oxygen species; $^1O_2$, singlet oxygen. Adapted from CALIXTO et al., (2016)

PDT is a procedure that consists of a combination of three components: the PS, light of a wavelength appropriate to excite the PS, and molecular oxygen. When the light excites the PS, it promotes oxidative mechanisms in two ways (Figure 5): the type I pathway involves electron transfer reactions from the activated state of the PS to a substrate, resulting in the production of free radicals which react with molecular oxygen to produce superoxide anion and radicals derived from lipids and hydroxyl. (reviewed by DĄBROWSKI, 2017). The type II pathway involves transferring energy from the excited state of the PS to molecular oxygen in the fundamental triplet state, producing excited singlet oxygen (reviewed by CASTANO; DEMIDOVA; HAMBLIN, 2005; DAI; HUANG; HAMBLIN, 2009).

The cytotoxic effect of singlet oxygen is due to its high reactivity with biomolecules such as proteins, nucleic acids and membrane lipids (DEMIDOVA; HAMBLIN, 2004). Especially in type II reactions, the photosensitizer molecule returns to its fundamental state, being able to continuously produce high levels of ROSs upon light exposure. In aerobic conditions, type II reactions are probably the main responsible for the oxidative damage caused by PDT in microbial cells (DONNELLY; MCCARRON; TUNNEY, 2008). After the PS binds to the cell wall, it can remain adsorbed or be translocated to the internal cell membrane or to the intracellular environment, where it will eventually exert the phototoxicity (FUCHS et al., 2007).

The application of PDT in the clinical practice of infection by microorganisms is still limited to the cutaneous infections (Reviewed by CIEPLIK et al., 2018). PDT is economically viable, generally safe, with demonstrated efficacy against different superficial fungal infections, but the effects are generally limited to the irradiated areas.

Generally, antimicrobial drugs target one specific site. So, pathogens can achieve resistance by modulating the drug target. PDT generates oxidative bursts, a non-selective reaction which affects multiple targets. Therefore, it is less likely that microbes attain resistance against PDT (Reviewed by CIEPLIK et al., 2018).

Chromoblastomycosis lesions were reduced after treatment with PDT employing 5-aminolevulinic acid (ALA) as PS, but they reappeared after the cessation of the PDT treatment (YANG et al., 2012). Later, HU and co-workers (2015) achieved complete eradication of the infection in one chromoblastomycosis patient, by combining ALA-mediated PDT in combination with terbinafine.

Recently, our research group demonstrated the diffuse internalization of PS, aluminum phthalocyanine chloride by *C. neoformans* H99 and *C. gattii* NIH198. In fluconazole-resistant

C. neoformans T1 strain, PS was restricted to the periphery of the cells. We also demonstrated that PDT was effective in controlling the growth of *Cryptococcus* spp., mainly when cells were pre-treated with the HDACi NaBut or TSA (MORAIS et al., 2020).

The selection of fungal isolates resistant to PDT is unlikely due to the multiplicity of target sites (MAISCH et al., 2004; KÖMERIK; MACROBERT, 2006). There are no reports in the scientific literature regarding the development of resistance to PDT by fungi (DONNELLY; MCCARRON; TUNNEY, 2008; DAI; HUANG; HAMBLIN, 2009). Therefore, PDT may represent an alternative treatment alone or in combination with epigenetic modulators or antifungals for superficial infections.

1.12 Photosensitizer

The ideal characteristics of a photosensitizer include chemical purity, absence of toxicity in the dark, phototoxicity, cell uptake and elimination, accumulation in the tissues of interest, activation in the wavelength of light that is highly penetrating in biological tissues, in addition to the ability to generate high amounts of reactive oxygen species (MUEHLMANN et al., 2015; ZHANG et al., 2018).

The photosensitizers most used in PDT include chlorins, porphyrins, phenothiazines and phthalocyanines. A PS that is gaining prominence in the scientific literature is aluminum phthalocyanine chloride (AlPcCl, Figure 6) due to its photochemical and photophysical properties. With a maximum absorption wavelength of 650-850 nm, light penetration is an important aspect in PDT, since light needs to reach the photosensitizer inside the cell. The light in the red region, which excites AlPcCl, has a penetrating power in biological tissues of about 3.0 mm (ZHANG et al., 2007).

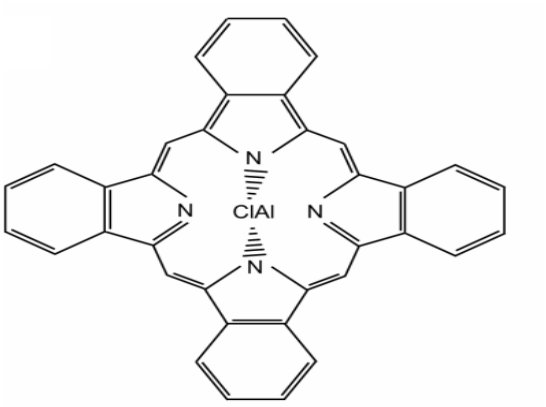


Figure 4: Representation of the chemical structure of aluminum phthalocyanine chloride (AlPcCl). Adapted from MORAIS, (2019)

Most PS, like AlPcCl and other phthalocyanines, are insoluble in water or in solvents compatible with biological systems. Thus, in the physiological environment, they tend to aggregate, which significantly reduces the photochemical properties of this photosensitizer (MUEHLMANN et al., 2015). In this context, the association of phototherapy with nanoparticulate systems improves the activity of PS in physiological environments (RODRIGUEZ et al., 2015). Some studies have demonstrated a greater effectiveness of the PS associated with nanoparticles both *in vitro* tests and *in vivo* models (GANASSIN et al., 2018). According to MUEHLMANN et al., (2014), the association of AlPcCl with polymeric nanoparticles resulted in greater efficiency of PDT in controlling breast cancer cells proliferation.

Among the different types of nanoparticles, the nanoemulsion stands out, as it is easy to obtain, produced with low cost components and is stable in media under different temperatures and conditions (LONGO et al., 2009). Nanoemulsions are colloidal systems capable of encapsulating, protecting and releasing lipophilic compounds and have been used in the pharmaceutical, food and cosmetic industry (MCCLEMENTS, 2011; BORRIN et al., 2016).

PDT was effective in the treatment of dermatophytes and black yeasts infections (YANG et al., 2012; HU et al., 2015). Therefore, PDT may represent an alternative approach to the cryptococcal cutaneous manifestation.

1.13 JUSTIFICATION

HDAC genes in *Cryptococcus* are associated with virulence attributes in *Cryptococcus* (BRANDÃO et al., 2015; BRANDÃO et al., 2018). Nonetheless, there is no report on the effect of HDAC genes deletion in the response to drugs. In this view, it is important to evaluate the pharmacological interactions between different combinations of traditional antifungal agents (amphotericin B, fluconazole, imidazole and sulfamethoxazole), epigenetic modulators (sodium butyrate, trichostatin A, hydralazine and 5-aza-2′deoxycytidine) and photodynamic therapy against *Cryptococcus*.

1.14 Objectives

a) To define the minimum inhibitory concentrations [(MICs), for antifungal drugs, epigenetic modulators and combinations of them] of *C. neoformans* H99 (reference strain), T1 and 89-610 (fluconazole-resistant strains), *C. gattii* NIH198, and HDAC null mutant strains (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ, *hda1*Δ+HDA1).

b) To evaluate the antimicrobial effect of photodynamic therapy (PDT) alone and in combination with antifungal drug or epigenetic modulators against the aforementioned *Cryptococcus* strains.

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The role of *Cryptococcus neoformans* histone deacetylase genes in the response to antifungal drugs, epigenetic modulators and to photodynamic therapy mediated by an aluminium phthalocyanine chloride nanoemulsion *in vitro*

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ABSTRACT

Cryptococcus is a globally distributed fungal pathogen that primarily afflicts immunocompromised individuals. The therapeutic options are limited and include mostly amphotericin B or fluconazole, alone or in combination. The extensive usage of antifungals allowed the selection of resistant pathogens posing threats to global public health. Histone deacetylase genes are involved in *Cryptococcus* virulence, and in pathogenicity and resistance to azoles in *Candida albicans*.

Aiming to assess whether histone deacetylase genes are involved in antifungal response and in synergistic drug interactions, we evaluated the activity of amphotericin B, fluconazole, sulfamethoxazole, sodium butyrate or trichostatin A (histone deacetylase inhibitors), and hydralazine or 5- aza-2′-deoxycytidine (DNA methyltransferase inhibitors) against different *Cryptococcus neoformans* strains, *C. neoformans* histone deacetylase null mutants and *Cryptococcus gattii* NIH198. The drugs were employed alone or in different combinations. Fungal growth after photodynamic therapy mediated by an aluminium phthalocyanine chloride nanoemulsion, alone or in combination with the aforementioned drugs, was assessed for the *C. neoformans* HDAC null mutant strains. Our results showed that fluconazole was synergistic with sodium butyrate or with trichostatin A for the *hda1*Δ/*hos2*Δ double mutant strain. Sulfamethoxazole was synergistic with sodium butyrate or with hydralazine also for *hda1*Δ/*hos2*Δ. These results clearly indicate a link between HDAC impairment and drug sensitivity. Photodynamic therapy efficacy on controlling the growth of the HDAC mutant strains was increased by amphotericin B, fluconazole, sodium butyrate or hydralazine. This is the first study in *Cryptococcus* highlighting the combined effects of antifungal drugs, histone deacetylase or DNA methyltransferase inhibitors and photodynamic therapy *in vitro*.

1. Introduction

Cryptococcus is an opportunistic fungal pathogen. In recent years, with the increase in the number of immunocompromised patients, cryptococcosis became one of the main systemic fungal infections. *Cryptococcus neoformans* and *Cryptococcus gattii* are the main species that cause this disease [1,2].

Fluconazole (FLU) and amphotericin B (AMB) are effective against most cryptococcal infections. In spite of being employed mainly against bacterial infections, sulfamethoxazole (SMX), a sulfa drug, has been used against *Aspergillus fumigatus* [3], *Cryptococcus* sp. [3,4], *Histoplasma capsulatum* [5], *Paracoccidioides lutzii* [6] *and Candida auris* [7].

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Histone acetylation and deacetylation play a key role in chromatin dynamics and are orchestrated by the activities of opposing effect enzymes: the histone acetyltransferases (HATs) and histone deacetylases (HDACs) [8]. *Cryptococcus* can adapt to environmental changes and to the specific cell stress conditions encountered during the interaction with host cells. Histone deacetylation-mediated chromatin remodeling is involved in this adaptation and in virulence [9,10].

The deletion of the *Candida albicans* histone deacetylase *HDA1 alleles* led to an increased susceptibility to azoles in comparison to the wild type strain [11]. The *HDA1* and *RPD3* genes also impacted the susceptibility of *C. albicans* to antifungal agents [12]. By gene deletion, we have reported that, amongst the seven HDAC genes identified in the *C. neoformans* genome, *HDA1* and *HOS2* are the most prominent for this fungus virulence attributes both *in vitro* as in animal models of infection [9,10]. Single and double mutant strains indicated that these two genes play distinct and overlapping roles and act as key regulators of *C. neoformans* virulence. Nonetheless, there is no study so far investigating whether histone deacetylase genes play some role in *C. neoformans* response to antifungal drugs.

Less than 1% of *Cryptococcus* strains were described as resistant to antifungals but, in general, drug resistance is increasing all over the world [13]. Drugs that can alter the epigenetic landscape, such as histone deacetylase inhibitors (HDACis) or DNA methyltransferase inhibitors (DNMTis), some of which have been used for cancer treatment, could be investigated as coadjutant in the treatment of infections provoked by strains which are resistant to traditional antifungal drugs.

Numerous studies in the scientific literature described sodium butyrate (NaBut) and trichostatin A (TSA) as potent histone deacetylases inhibitors. Different techniques, such as HPLC analysis of purified radiolabelled histones [14], immunohistochemistry and immunoprecipitation [15], fluorescent signalling of enzyme-substrate interaction [16] and Western blot [17] demonstrated the deacetylase activity inhibition in diverse cell types.

Nguyen and colleagues demonstrated that NaBut affected *C. albicans* and *C. neoformans* growth and virulence traits, and increased the antifungal activity of a murine macrophage cell line [18] Our research group reported that NaBut or TSA impaired *C. neoformans* virulence phenotypes as thermotolerance, the expansion of the polysaccharide capsule, melanisation, formation of mating hyphae, phospholipase activity and the progression in cell cycle *in vitro* [9]. Recently, Cordeiro and coworkers (2019) showed that NaBut inhibited the growth of planktonic cells and reduced the filamentation, the metabolic activity and the biofilm formation in *Trichosporon* spp. opportunistic pathogens [17].

In *C. albicans*, 5′-azacytidine, a DNMTi, synchronized and accelerated the germ tubes formation during the yeast-mycelium transition; this suggested that DNA demethylation is important for the regulation of the dimorphic transition associated with virulence [19]. In *A. nidulans*, 5-azacytidine induced the abnormal fluffy phenotype, and delayed conidia formation [20]. Lower risk of invasive fungal infections was reported when acute myeloid leukaemia and myelodysplastic syndromes patients were treated with 5-azacytidine [21].

Hydralazine was approved by the U.S. Food & Drug Administration (FDA) for human blood pressure control in 1985. It was then reported as a DNMTi which could restore the expression of tumor suppressor genes in cancer cells [22–24]. There are a few studies on the ability of hydralazine to modulate fungal secondary metabolism genes aiming the obtainment of new bioactive compounds [25,26], but a possible effect on fungal growth was not yet investigated.

Photodynamic therapy (PDT) involves a photosensitizer agent (PS) which is activated by a suitable light wavelength generating reactive oxygen species. This therapy inhibited cancer cells proliferation *in vitro* [27] and *in vivo* [28]. It has been used in the treatment of bacteria, viruses and parasites infections [29]. Nonetheless, the application of PDT to control fungal growth is limited to dermatophytes and to black yeasts affections [30]. So far, there are no reports on the development of resistance to PDT by microbes [31]. Therefore, PDT can represent an alternative therapy to the cutaneous manifestation of *Cryptococcus* infection [32].

Here, we evaluated the role of *C. neoformans* histone deacetylase genes in the response to antifungal drugs, to epigenetic modulators and to PDT, employed alone or in different combinations.

2. Materials and Methods

2.1. Strains and Growth Conditions

C. neoformans H99, the fluconazole-resistant 89–610 and T1 strains [33] and *C. gattii* NIH 198 were generously provided by Dr. Joseph Heitman Laboratory (Duke University Medical Center, Durham-USA). The *hda*1Δ, *hos*2Δ, *hda*1Δ/*hos*2Δ and the reconstituted strain *hda*1Δ + HDA1 strains were generated and phenotypically characterized by our group [10]. The strains were stored as 35% glycerol stocks at −80 °C and grown on YPD agar plates at 30 °C.

2.2. MICs Determination by Broth Microdilution Assays

AMB, FLU, SMX and HLZ were purchased from Sigma Aldrich® (St. Louis, MO, USA). NaBut, TSA, and 5-aza-2′-deoxycytidine (5-AzadC) were purchased from Cayman Chemical Company (Michigan, USA). Stock solutions of antifungal drugs were prepared as recommended by the NCCLS M27-A protocol [34] and the epigenetic modulator drugs as per manufacturer's instructions. NaBut and HLZ were dissolved in Milli-Q water, while TSA and 5-AzadC were dissolved in DMSO. The drugs were tested in the following range against all strains: AMB, 0.03 to 16 μg mL^{-1}; FLU, 0.125 to 64 μg mL^{-1}; SMX, 0.156 to 79.87 mg mL^{-1}; NaBut, 1 to 512 mM; TSA, 0.25 to 128 μM; HLZ, 0.02 to 12.5 mM and 5-AzadC, 0.065 to 1 mM.

All the strains were grown in YPD broth and incubated for 20 h at 30 °C with shaking. Cells were washed with 1× PBS and the inoculum concentration was adjusted to 4×10^4 cells mL^{-1} in 2× RPMI 1640 media with L-glutamine and without sodium bicarbonate (Sigma Aldrich®, St. Louis, MO, USA), buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). One hundred μL of this solution was added to the wells containing 100 μL of the 2× concentrated drug. Positive controls without drugs and negative controls without yeast cells were performed. Plates were protected from light and incubated at 37 °C, to mimic mammal body temperature. After 72 h of incubation, endpoints were visually read. MIC concentration was defined as the lowest concentration that completely abolished fungal growth.

2.3. Evaluation of Synergism

The possible synergism between antifungal drugs and epigenetic modulators was determined by the checkerboard method [35]. The drug concentrations varied from five-fold dilutions below to four-fold above the estimated MIC.

Microbial growth was evaluated as above and fractional inhibitory concentration (FIC) was calculated as previously described by Pfaller and colleagues [35]. FIC was defined as the quotient of the division between the MIC of the drugs employed in combination divided by the MIC of each drug tested alone. FIC index (FICI) was calculated as the sum of the FICs of each drug present in the assay. The result of the FICI was interpreted as follows: ≤ 0.5 - synergy, > 0.5 and ≤ 4.0 - indifferent, > 4.0 – antagonism [35].

2.4. Photodynamic Therapy

To study the PDT effect alone, cells (1×10^5 mL^{-1}) were incubated with increasing concentrations of the PS, *i.e.* aluminium-phthalocyanine chloride nanoemulsion (AlPcCl). [3.15; 6.25; 12.50; 25.00; 50.00; 100.00; 200.00 or 400.00 nM] for 30 min at 30 °C, with shaking at 50 rpm, in the dark, to allow PS absorbing, as previously standardized in

Table 1
Minimum inhibitory concentration (MIC) values for the antifungal drugs and the epigenetic modulators, employed separately, for the *C. neoformans* H99, T1 and 89-610 and *C. gattii* NIH198 strains.

	MICs						
	Antifungal drugs			Epigenetic modulators			
	AMB (μg mL^{-1})	FLU (μg mL^{-1})	SMX (mg mL^{-1})	NaBut (mM)	TSA (μM)	HLZ (mM)	5-AzadC (mM)
C. neoformans H99	0.5	4	0.625	128	32	0.39	0.25
C. neoformans T1	0.5	8	1.25	32	16	0.79	0.25
C. neoformans 89-610	0.25	16	0.625	64	32	0.39	0.25
C. gatti NIH198	0.5	8	0.625	64	32	0.39	0.25

MIC: Minimum inhibitory concentration, AMB: Amphotericin B, FLU: Fluconazole, SMX: Sulfamethoxazole, NaBut: Sodium butyrate, TSA: Trichostatin A, HLZ: Hydralazine and 5-AzadC: 5-aza-2′-deoxycytidine.

our group [36]. The interaction between PDT, antifungal drugs (AMB, FLU or SMX), HDACi (NaBut), or DNMTi (HLZ) was evaluated. Yeasts were firstly exposed to different concentrations of drugs and then exposed to PDT, as it was described for the evaluation of synergism between antifungal drugs and PDT for controlling the *Fonsecaea monophora* infection *in vivo* [30]. Cells were exposed to 50 or 70% of the MIC value for each antifungal drug, for 48 h at 30 °C, 50 rpm, and exposed to PDT. The same experimental design was employed for HLZ. For NaBut, 10 and 20% of the MIC were employed, since higher concentrations would result in not enough cells to perform the experiment.

For the evaluation of the antifungal drugs (AF), HLZ and PDT interaction, the following combinations were assayed: 1) 50% of the AF or 50% of the HLZ MIC +50% of the PS MIC (50% AF or HLZ + 50% PS). Likewise, 2) (50% AF or HLZ + 75% PS), 3) (75% AF or HLZ + 50% PS) or 4) (75% AF or HLZ + 75% PS). For NaBut, 1) (10% NaBut+50% PS), 2) (10% NaBut+75% PS) 3) (20% NaBut+50% PS) or 4) (20% NaBut+75% PS). Controls consisted of (1) cells not previously exposed to the drugs but which were incubated with PS; (2) cells exposed to the drugs but not to PS and (3) cells that were not exposed to drugs or to PS (positive control). Cells were incubated at 30 °C, for 48 h, with shaking.

After incubation, cells were washed with 1× PBS. One hundred μL of the cell solution was added to the wells in two different 96 well plates. One plate was kept in the dark, while the other one was irradiated with a 660 nm LED apparatus for 30 min (final energy density of 107 J cm^{-2}). The LED equipment was developed by Prof. Paulo Eduardo Narciso de Souza and collaborators (Laboratory of Software and Instrumentation of the Institute of Physics of the University of Brasilia, Brasilia, Brazil). One hundred μL of 2× RPMI medium was then added to the cells which were incubated at 37 °C for 72 h, under orbital shaking, in the Epoch 2 EON Microplate reader (Biotek Inc., USA). Cell proliferation was assessed by measuring the absorbance at 600 nm every 30 min during 72 h. The effect of PDT on the cell proliferation was assessed after 72 h as commonly observed in the *Cryptococcus* literature.

2.5. Statistical Analysis

All the MIC assays and PDT experiments were conducted as three independent experiments with three technical replicates. Checkerboard assays and PDT in combination with drugs evaluations were performed as three independent experiments. Graphs and statistical analyses were performed with the GraphPad Prism 6.0 software (California, USA), with 0.05 level of significance. The error bar represents the standard deviation. Synergism data were presented as tile plots generated by the geom_raster function from the ggplot2 package [37] of R programming. The full reproducible code is presented as supplementary material (TilePlot_R code).

3. Results

3.1. Determination of MICs

The susceptibility to all the drugs for the *C. neoformans* H99, T1 and 89–610 strains, and *C. gattii* NIH198 strain is shown in Table 1. MICs ranged from 0.25 to 0.5 μg mL^{-1} for AMB, 4 to 16 μg mL^{-1} for FLU, 0.625 to 1.25 mg mL^{-1} for SMX, 32 to 128 mM for NaBut, 16 to 32 μM for TSA, 0.39 to 0.79 mM for HLZ and 0.25 mM for 5-AzadC.

Considering H99 as the reference strain, the MIC values obtained for AMB and FLU were within the range previously reported for different *C. neoformans* clinical isolates [38,39]. The *C. neoformans* 89–610 strain was more susceptible to AMB in comparison to H99 and the T1 strains. *C. gattii* NIH 198 presented similar susceptibility to AMB when compared to H99.

We confirmed the T1 and 89–610 strains as fluconazole resistant [33]; MIC values were determined as 8 and 16 μg mL^{-1}, respectively (Table 1). The *C. gattii* NIH 198 strain was more resistant to FLU (8 μg mL^{-1}) than *C. neoformans* H99 (4 μg mL^{-1}). The *C. neoformans* H99 yeasts were the most resistant to SMX (1.25 μg mL^{-1}). The inhibitory values we observed for this antibiotic were higher than those reported by Cordeiro and colleagues, for environmental and clinical strains of *C. neoformans* and *C. gattii* [4].

Table 2
Minimum inhibitory concentration (MIC) values for the antifungal drugs and the epigenetic modulators, employed separately, for the *C. neoformans* H99 and for the *C. neoformans* histone deacetylase null mutants.

	MICs						
	Anti-fungal drugs			Epigenetic modulators			
	AMB (μg mL^{-1})	FLU (μg mL^{-1})	SMX (mg mL^{-1})	NaBut (mM)	TSA (μM)	HLZ (mM)	5-AzadC (mM)
C. neoformans H99	0.5	4	0.625	128	32	0.39	0.25
*hda1*Δ	0.06	2	0.625	256	16	0.19	0.25
*hos2*Δ	0.125	2	0.625	256	8	0.39	0.25
*hda1*Δ/*hos2*Δ	0.125	4	0.312	128	8	0.19	0.25
*hda1*Δ + HDA1	0.125	4	0.625	256	32	0.39	0.25

MIC: Minimum inhibitory concentration, AMB: Amphotericin B, FLU: Fluconazole, SMX: Sulfamethoxazole, NaBut: Sodium butyrate, TSA: Trichostatin A, HLZ: Hydralazine and 5-AzadC: 5-aza-2′- deoxycytidine.

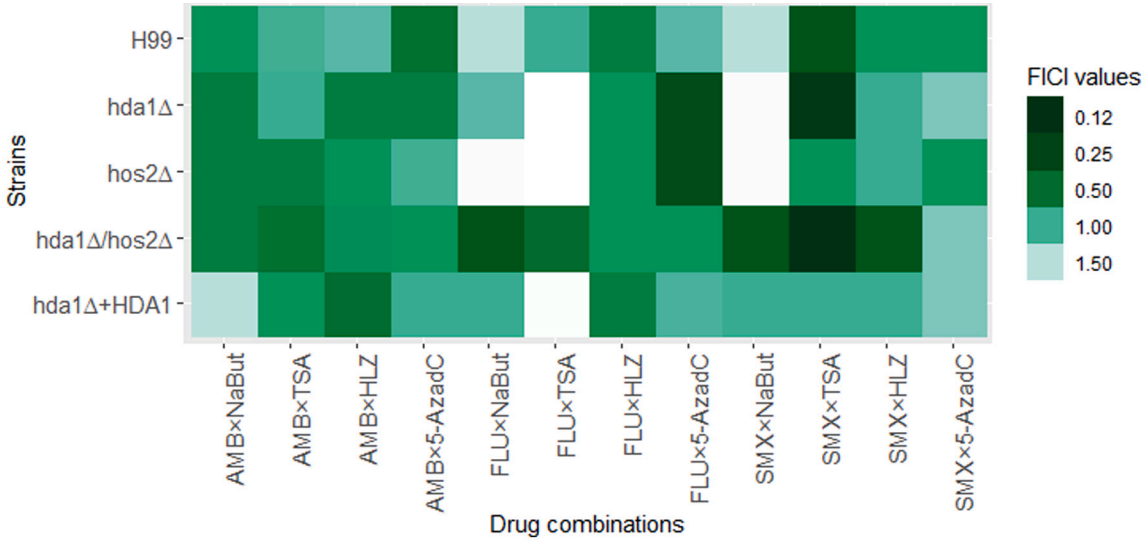


Fig. 1. Tile plot representation of the fractional inhibitory concentration indexes (FICIs) for the combinations of antifungal drugs and epigenetic modulators for *C. neoformans* H99 and HDAC mutant strains (*hda1*Δ, *hos2*Δ, *hda1*Δ/ *hos2*Δ and *hda1*Δ + HDA1). FICI ≤0.5 = synergy; > 0.5 and ≤ 4.0 = indifferent; > 4.0 = antagonism [24]. AMB: Amphotericin B, FLU: Fluconazole, SMX: Sulfamethoxazole, NaBut: Sodium butyrate, TSA: Trichostatin A, HLZ: Hydralazine and 5-AzadC: 5-aza-2′-deoxycytidine.

C. neoformans T1 and 89–610 strains, as well as *C. gattii* NIH198, were more susceptible to NaBut than *C. neoformans* H99. Except for *C. neoformans* T1, which was more susceptible (16 μM), the other *C. neoformans* strains, and also *C. gattii*, responded similarly to TSA, tolerating up to 32 μM.

Considering the DNMTi, *C. neoformans* T1 was more resistant to HLZ than all the *C. neoformans* strains and *C. gattii* NIH 198. No difference in the sensitivity to 5-AzadC was observed amongst the distinct yeasts.

The MIC values for the gene deletion null mutants in comparison to the H99 strain are presented in Table 2. MICs ranged from 0.06 to 0.5 μg mL^{-1} for AMB, 2 to 4 μg mL^{-1} for FLU, 0.312 to 0.625 mg mL^{-1} for SMX, 128 to 256 mM for NaBut, 8 to 32 μM for TSA, 0.19 to 0.39 mM for HLZ and 0.25 mM for 5-AzadC. The reconstituted strain *hda1*Δ + HDA1 exhibited similar results to the H99 reference strain, unless otherwise stated.

Chromatin remodeling genes mutant strains were shown to be more susceptible to AMB, particularly *hda1*Δ (0.06 μg mL^{-1}). For this drug, the *hda1*Δ + HDA1 reconstituted strain was more susceptible (0.125 μg mL^{-1}) than H99 (0.5 μg mL^{-1}). Increased susceptibility to FLU was detected for the *hda1*Δ and *hos2*Δ strains (MIC 2 μg mL^{-1}). The *hda1*Δ/*hos2*Δ double mutant was more sensitive to SMX (0.312 mg mL^{-1}) than all the other strains (0.625 mg mL^{-1}).

In regard to the epigenetic modulators, both single-gene deletion strains (*hda1*Δ and *hos2*Δ) and the *hda1*Δ + HDA1 reconstituted strain tolerated a higher NaBut concentration (256 mM) than the double mutant *hda1*Δ/*hos2*Δ (128 mM), which was as susceptible as H99. On the other hand, susceptibility to TSA increased by two-fold in *hda1*Δ (16 μg mL^{-1}) and by four-fold in *hos2*Δ and *hda1*Δ/*hos2*Δ null mutants (8 μg mL^{-1}). Only the *hda1*Δ and the *hda1*Δ/*hos2*Δ strains were more susceptible to HLZ (0.19 mM). For the other DNMTi, 5-AzadC, no difference in susceptibility was observed amongst all strains.

3.2. Synergism

For the antifungal drugs and epigenetic modulators, no synergism was observed for *C. neoformans* H99, T1, and 89–610 strains; neither for *C. gattii* NIH198 (Supplementary Table S1).

Nonetheless, the gene deletion strains presented synergism for certain drug combinations (Fig. 1). The FICI values are provided in Supplementary Table S2. No antagonistic interaction for any drug combination was detected for the *Cryptococcus* strains.

Amphotericin B did not present synergistic interaction with any of the epigenetic modulators for any mutant strain. FLU was synergistic with NaBut (FICI = 0.37) and with TSA (FICI = 0.50) only for the *hda1*Δ/*hos2*Δ double mutant.

The double mutant *hda1*Δ/*hos2*Δ strain also presented synergism for SMX with NaBut (FICI = 0.25) or with HLZ (FICI = 0.37). SMX was also synergistic with TSA for the H99 (0.37), *hda1*Δ (FICI = 0.18) and *hda1*Δ/*hos2*Δ (FICI = 0.12) strains. In contrast to H99, the *hda1*Δ + HDA1 reconstituted strain did not present synergism to the SMX and TSA drug combination.

3.3. Photodynamic Therapy (PDT) Effect on Cryptococcus Growth

The response of *C. neoformans* H99, T1, 89–610, and *C. gattii* NIH198 to PDT was previously reported by our group [36]. In this study, we evaluated the effect of PDT on the *C. neoformans* gene deletion *hda1*Δ, *hos2*Δ, and *hda1*Δ/*hos2*Δ mutant strains (Fig. 2).

No effect of the PS was observed without irradiation. In contrast, a PS concentration-dependent growth impairment was observed after exposure to light. Cell proliferation of the H99, *hos2*Δ, and *hda1*Δ + HDA1 strains was completely abolished from the 12.5 nM PS concentration (Fig. 2 a, c, e). The *hda1*Δ and *hda1*Δ/*hos2*Δ mutants were more sensitive to PDT, since growth abolishing occurred from 6.25 nM PS (Fig. 2 b, d).

3.4. The Combined Effect of Photodynamic Therapy, Antifungal, and Epigenetic Drugs on the Proliferation of C. neoformans H99 and HDAC Null Mutant Strains

As shown in Fig. 3, when compared to the positive control (no drug and no PS but exposed to light) (Fig. 3), all the four AMB + PS combinations reduced cell growth to the same level of 100% PS for the *hda1*Δ null mutant. On the other hand, only the (50% AMB + 75% PS) and (75% AMB + 75% PS) combinations resulted in such effect for the H99, *hos2*Δ, *hda1*Δ/*hos2*Δ, and *hda1*Δ + HDA1 strains.

When PDT was applied after the exposure to FLU, all the strains showed significantly reduced growth, regardless of the FLU+PS combination (Fig. 4). Only the 75% FLU+75% PS combination completely abolished growth.

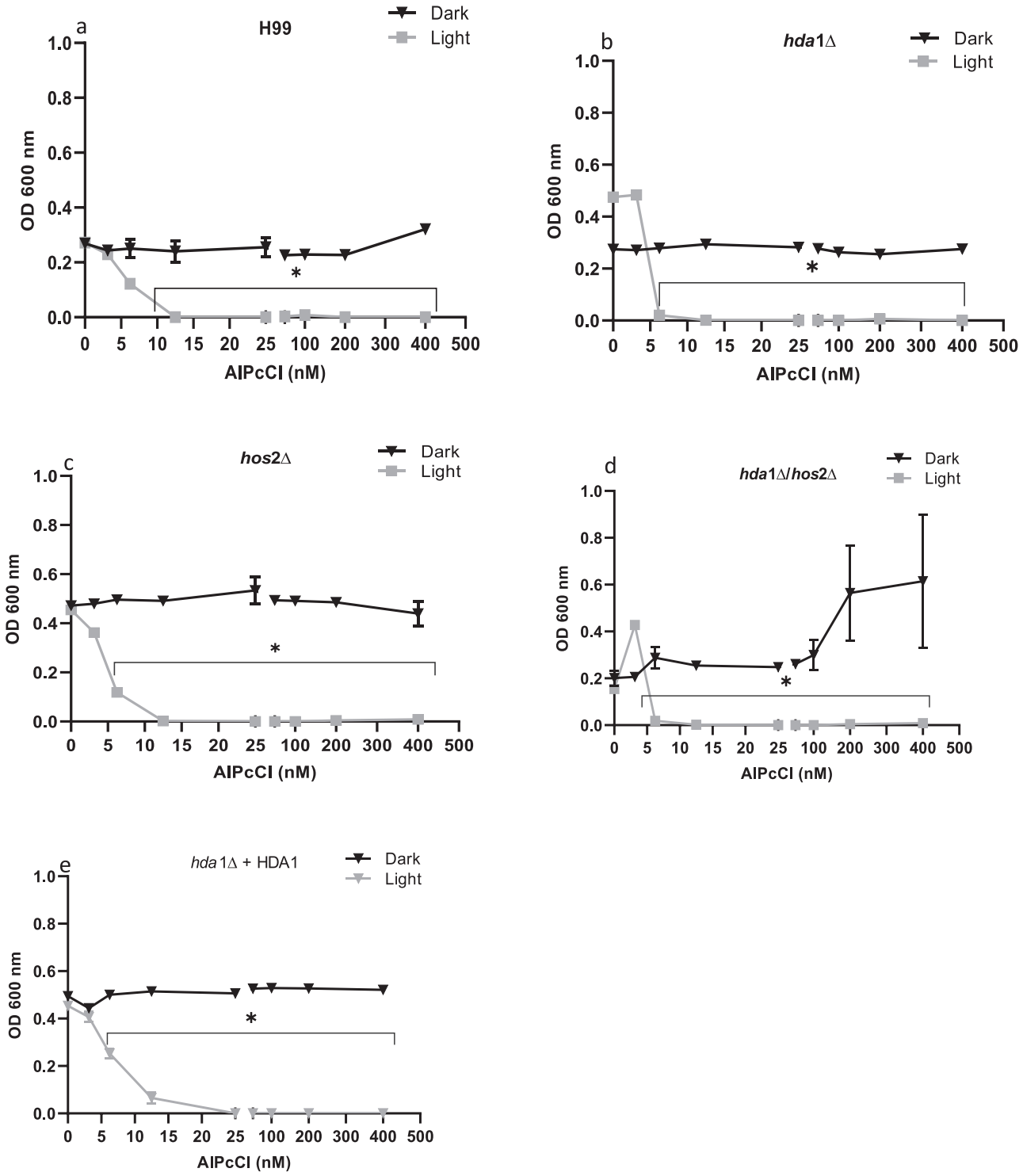


Fig. 2. Growth curve for *C. neoformans* a) H99, b) *hda1*Δ, c) *hos2*Δ, d) *hda1*Δ/*hos2*Δ, and e) *hda1*Δ + HDA1 strains after exposure to different concentrations of aluminium phthalocyanine chloride (AlPcCl) nanoemulsion in the dark (▼) and irradiation (■). The Y-axis indicates the absorbance value at 600 nm after 72 h. (*) <0.01 corresponds to statistically significant difference from the control, determined by two-way ANOVA and multiple comparisons by Tukey test.

When the yeast cells were exposed to SMX and then to PDT, no growth inhibition was detected, irrespectively of the strain and the combinations (Supplementary Fig. S1).

The combination of NaBut and PDT resulted in similar results for the H99, *hos2*Δ, and *hda1*Δ/*hos2*Δ strains: only the 20% NaBut + 75% PS combination resulted in a significant reduction in cell proliferation. On the other hand, for the *hda1*Δ strain, two different combinations [(20% NaBut + 75% PS) and (10% NaBut + 75% PS)] significantly reduced growth. No complete growth inhibition was observed for any mutant strain, no matter the NaBut + PS combination (Fig. 5).

When *C. neoformans* H99, *hda1*Δ, *hos2*Δ, and *hda1*Δ + HDA1 strains were previously exposed to HLZ, the combinations (75% HLZ + 75%

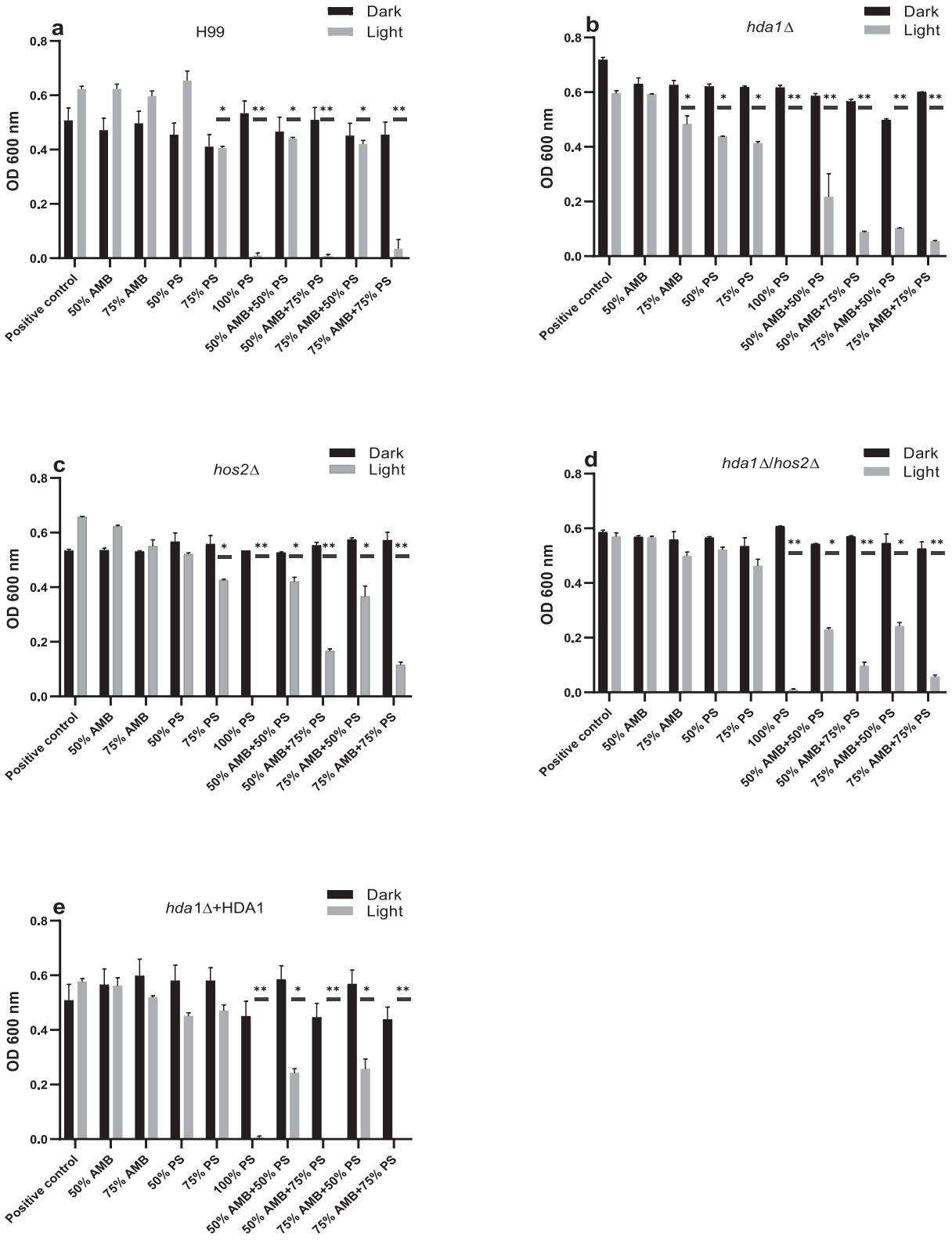


Fig. 3. Bar graph representation of the effect of photodynamic therapy (PDT) mediated by different combinations of aluminium phthalocyanine chloride (AlPcCl) nanoemulsion [PS] and amphotericin B (AMB) on the proliferation of *C. neoformans* a) H99, b) *hda*1Δ, c) *hos*2Δ, d) *hda*1Δ/*hos2*Δ and e) *hda*1Δ + HDA1 strains. The Y-axis indicates the absorbance value at 600 nm after 72 h. dark (—), light ( ). (*) <0.01 and (**) <0.001 correspond to statistically significant difference from the control, determined by two-way ANOVA and multiple comparisons by Dunnett's test. 50% PS indicates 6.25 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 3.13 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ, 75% PS indicates 9.38 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 4.69 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ and 100% PS indicates 12.5 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 6.25 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ.

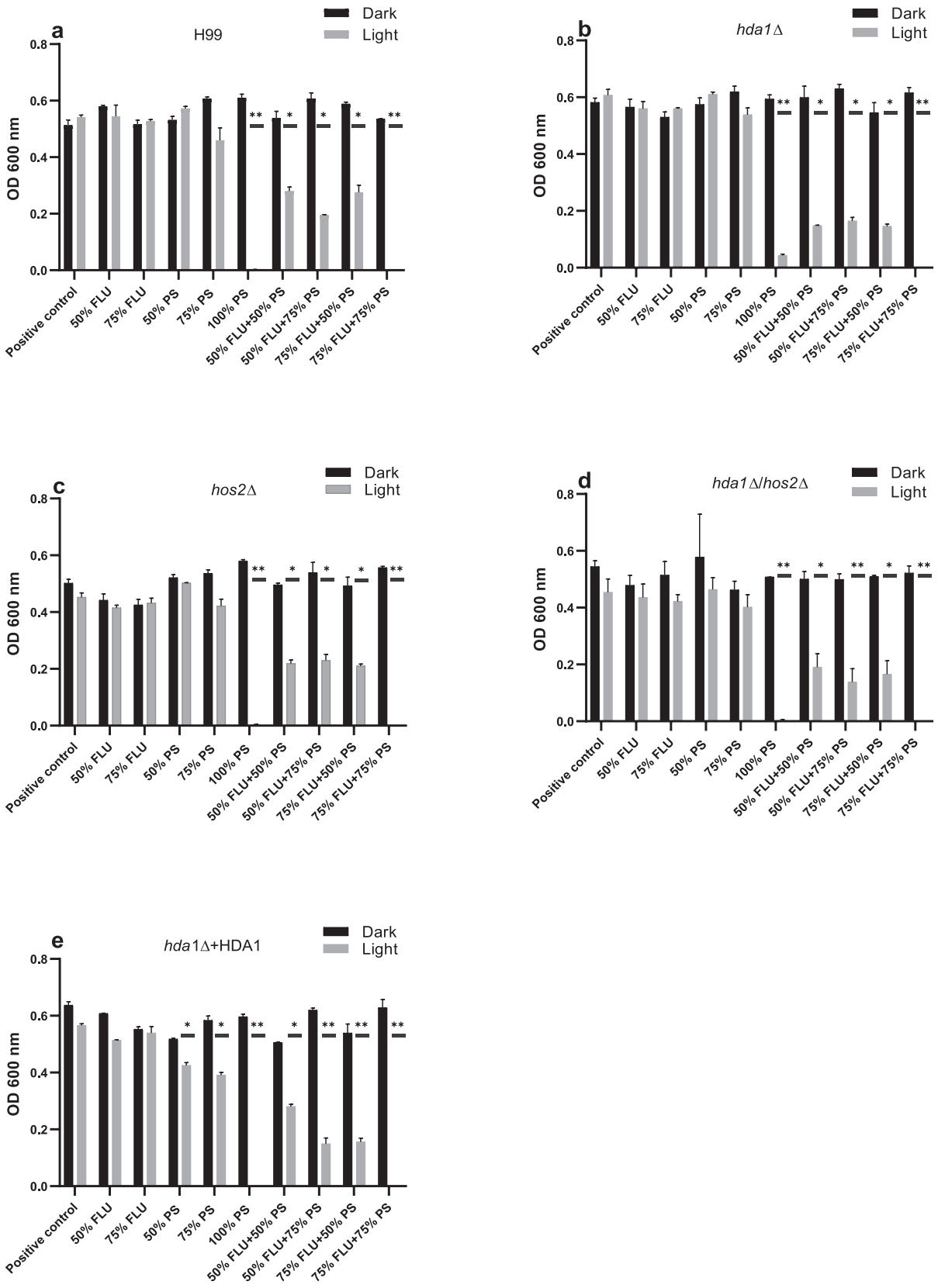
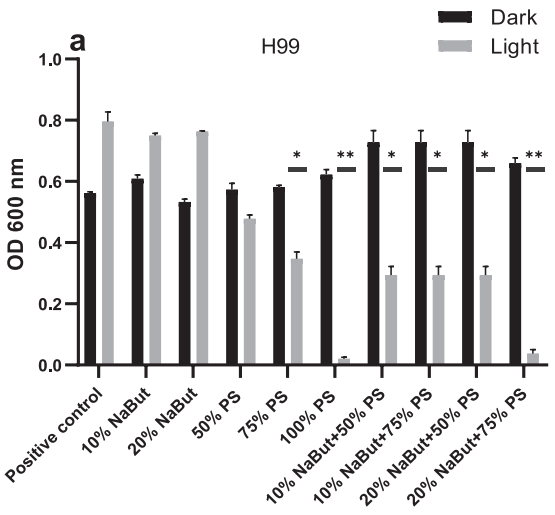
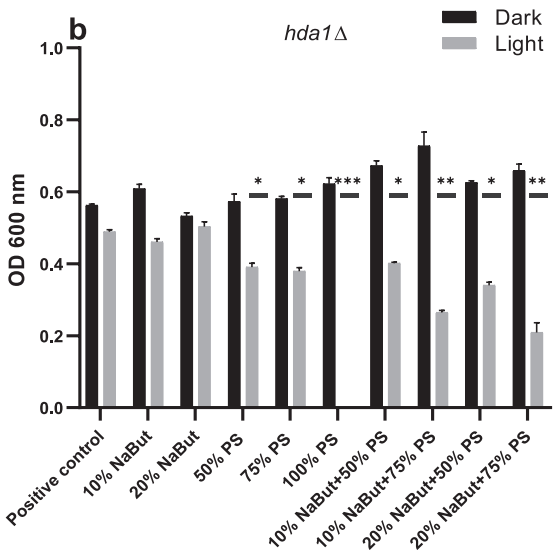
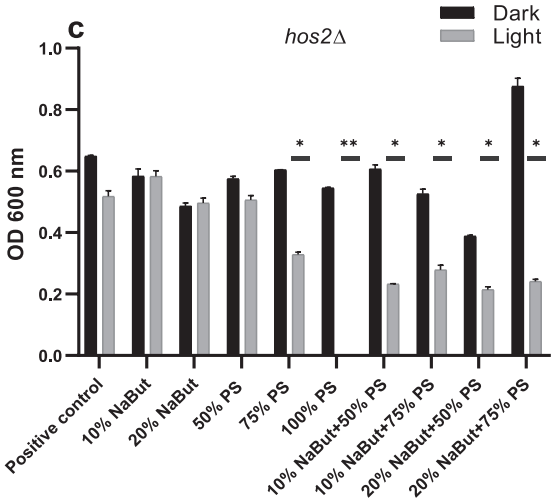
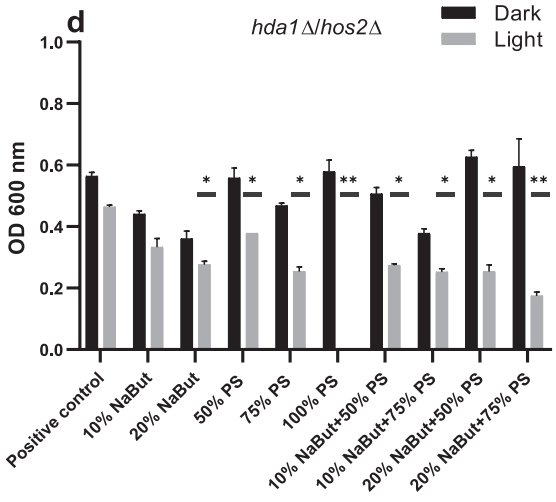


Fig. 4. Bar graph representation of the effect of photodynamic therapy (PDT) mediated by different combinations of aluminium phthalocyanine chloride (AlPcCl) nanoemulsion [PS] and fluconazole (FLU) on the proliferation of *C. neoformans* a) H99, b) *hda*1Δ, c) *hos*2Δ, d) *hda*1Δ/*hos2*Δ and e) *hda*1Δ + HDA1 strains. The Y-axis indicates the absorbance value at 600 nm after 72 h. dark (—), light (▬). (*) <0.01 and (**) <0.001 correspond to statistically significant difference from the control, determined by two-way ANOVA and multiple comparisons by Dunnett's test. 50% PS indicates 6.25 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 3.13 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ, 75% PS indicates 9.38 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 4.69 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ and 100% PS indicates 12.5 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 6.25 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ.









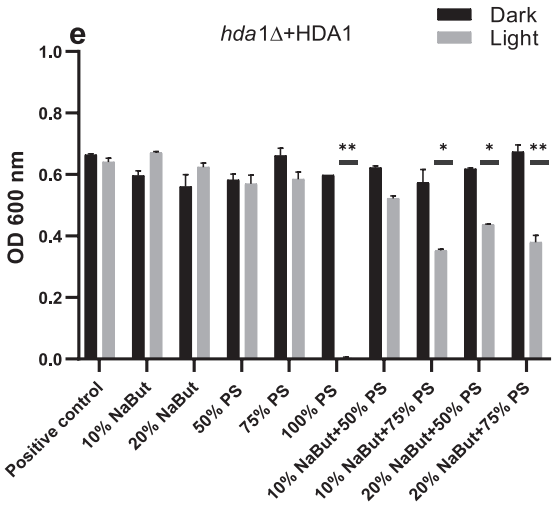


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Fig. 5. Bar graph representation of the effect of photodynamic therapy (PDT) mediated by different combinations of aluminium phthalocyanine chloride (AlPcCl) nanoemulsion [PS] and sodium butyrate (NaBut) on the proliferation of *C. neoformans* a) H99, b) *hda*1Δ, c) *hos*2Δ, d) *hda*1Δ/*hos2*Δ and e) *hda*1Δ + HDA1 strains. The Y-axis indicates the absorbance value at 600 nm after 72 h. dark (—), light ( ). (*) <0.01, and (***) <0.0001 correspond to statistically significant difference from the control, determined by two-way ANOVA and multiple comparisons by Dunnett's test. 50% PS indicates 6.25 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 3.13 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ, 75% PS indicates 9.38 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 4.69 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ and 100% PS indicates 12.5 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 6.25 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ.

PS), (50% HLZ + 75% PS) and (75% HLZ + 50% PS) significantly reduced the cell growth to the 100% PS level. The (50% HLZ + 50% PS) combination reduced the yeasts proliferation to a similar level of the one observed when HLZ (50 or 75% of MIC) or PS (75% of MIC) were tested alone. In contrast, for the *hda*1Δ/*hos*2Δ double mutant strain, all the HLZ + PS combinations significantly reduced cell growth similarly to 100% PS (Fig. 6).

4. Discussion

Cryptococcus causes severe infection in immunocompromised hosts. There is an increasing concern about the infection of immunocompetent individuals, particularly for *C. gattii* [1], and also on the selection of drug-resistant clinical isolates [13]. The aim of this study was to evaluate the combined effect of antifungal drugs (AMP, FLU or SMX) with epigenetic modulators (NaBut, TSA, HLZ or 5-Azadc) and the combination of different drugs with PDT, in the search of the most effective strategy to control superficial *C. neoformans* infections with the lowest number of components. Our research model employs *C. neoformans* fluconazole resistant and HDAC mutant strains to evaluate a possible link amongst drug resistance, epigenetic regulation and PDT-induced oxidative stress on fungal growth control.

In the present work, *C. neoformans* FLU-resistant strains (T1 and 89–610) were reported as more susceptible to HDACis when compared to the H99 reference strain (Table 1). This result is consistent with a *C. albicans* study, which demonstrated that TSA reduced the cell growth rate, *in vitro*, for azole-resistant clinical isolates, as well as for the resistant ATCC 64550 strain [11]. These authors also showed that FLU susceptibility is increased in the HDAC null mutant strains (HDho15 and RPho3). The deletion of HDAC genes resulted in a 16 times reduction of trailing growth with FLU. Increased susceptibility to FLU was also observed for the *C. neoformans hda1*Δ and *hos2*Δ strains (Table 2).

Single HDAC gene deletion strains (*hda*1Δ, *hos*2Δ) were less susceptible to NaBut when compared to the *hda*1Δ/*hos*2Δ double mutant strain (Table 2). Curiously, for TSA, the double mutant tolerated a higher concentration than the single gene mutants (Table 2). We believe the different results observed for NaBut and TSA corroborate the distinct yet redundant roles of *C. neoformans* Class I and II HDACs proposed by Brandão et al. (2018) [10].

Even though we did not report synergism between antifungal drugs and epigenetic modulators for *C. neoformans* H99, T1, or 89–610 strains, neither for *C. gattii* NIH198 (Supplementary Table S1), a clear synergic interaction was observed for the *C. neoformans* HDAC mutant strains.

The *hda*1Δ/*hos*2Δ strain showed synergism for FLU in combination with NaBut and with TSA (Fig. 1, Supplementary Table S2). Robbins and colleagues demonstrated that Hsp90 impairing by TSA markedly reduced the resistance of *C. albicans* clinical isolates to azoles [12]. It is possible that the stronger effect of FLU in the presence of HDACi in the *hda*1Δ/*hos*2Δ double mutant reflects the severe commitment of the HDAC machinery.

Rajasekharan and colleagues (2015) reported that NaBut and the flavonoids quercetin and kaempferol, when employed alone, did not show antifungal or antibiofilm activity on *Candida tropicalis*. Nonetheless, the combined utilization of NaBut and the flavonoids significantly reduced the biofilm formation [40]. These data illustrate the potential of HDACi to act synergistically with other classes of compounds in controlling fungi proliferation and virulence traits.

To date, the U.S. FDA has approved three HDACis for the treatment of cutaneous and/or peripheral T-cell lymphomas (vorinostat, romidepsin and belinostat) [41]. The Hos2 inhibitor MGCD290, developed by MethylGene, Inc., Montreal, Canada, presented promising results in controlling *Candida* spp. and other fungi proliferation when combined with azoles and echinocandins *in vitro* and in murine models of infection [42]. It was tested in combination with fluconazole, against vulvovaginal candidiasis in a clinical phase 2 trial (NCT01497223). Nonetheless, no statistically significant effect on fungal growth was observed when compared to fluconazole alone [43].

Fournel and colleagues [44] demonstrated that sulfonamide anilides sulfa drugs inhibited the function of a partially purified recombinant human HDAC1 and could control cell growth in human tumors. SMX alone and in combination with trimethoprim (TMP) strongly inhibited *Cryptococcus* sp. growth and biofilm formation by reducing the ergosterol content. The SMX-TMP combined action also rendered the free cells more sensitive to amphotericin B [3,4]. In our study, we detected synergism between SMX and NaBut or TSA for the *hda*1Δ/*hos2*Δ double mutant strain, but not for the single-gene mutants (Fig. 1, Supplementary Table S2). SMX was also synergistic with HLZ, a DNMTi, only in the *hda*1Δ/*hos*2Δ double mutant (Fig. 1, Supplementary Table S2). SMX showed potent synergy with azoles against the multidrug resistant *C. auris* emergent pathogen [7]. We hypothesize that NaBut, TSA, or HLZ, which alter chromatin configuration, led to a more open chromatin state that favours the action of SMX in the *hda*1Δ/*hos*2Δ genetic background.

Our data indicate a clear link between HDAC impairment (both by chemical inhibitors and the gene deletion strains) and increased drug susceptibility in *Cryptococcus*.

The more pronounced effects observed for the *hda*1Δ/*hos*2Δ double mutant shall reflect the distinct and redundant roles we previously described for HDAC genes in *C. neoformans* virulence [10].

Several studies addressed PDT employing different PSs and irradiation parameters for reducing cancer cells proliferation *in vitro* [27,45] On the other hand, there is a scarcity of data on this therapy for inhibiting pathogenic yeasts.

de Santi and collaborators demonstrated that PDT was efficient in treating BALB-c mice with vaginal candidiasis [46]. These authors tested two PS: methylene blue and protoporphyrin IX. Both presented fungicide effect and reduced the mucosa swelling in the animals.

The PS we employed in our experiments (AlPcCl) had been successfully used for PDT in the treatment of cutaneous hemangiosarcoma in dogs [47]. Recently, we demonstrated that AlPcCl-mediated PDT was effective in controlling *C. neoformans* H99 and T1 strains and *C. gattii* NIH 198 growth, particularly when combined with the HDACi NaBut or TSA [36]. In the present study, we evaluated the combination of different antifungal drugs, NaBut, or the DNMTi hydralazine, with AlPcCl-mediated PDT in the proliferation of the *C. neoformans* H99 reference strain and in HDAC null mutants.

We observed that AMB, FLU, NaBut (HDACi), or HLZ (DNMTi), potentiated the antimicrobial activity of PDT in all the strains. Previously, photodynamic therapy in combination with itraconazole, terbinafine, or voriconazole was successfully employed for the treatment of human chromoblastomycosis [30,48].

Noguichi and colleagues [32] pointed out that, even not being much frequent, localized cutaneous cryptococcosis, is relevant to the dermatology practise. These authors revealed, by Giemsa and India ink staining, the presence of encapsulated yeasts in discharged smears from cutaneous cryptococcosis patients skin lesions. Chakradeo and

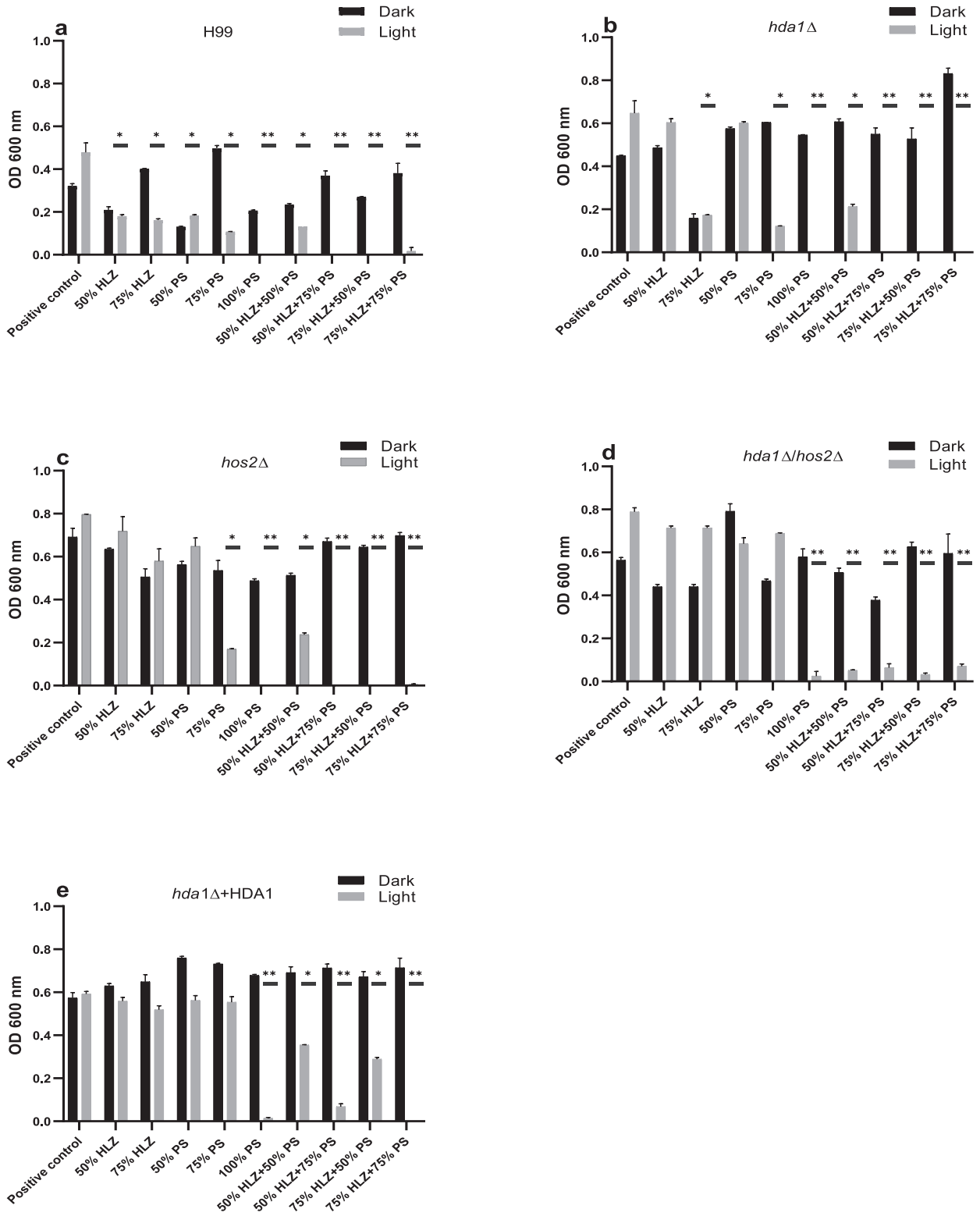

Fig. 6. Bar graph representation of the effect of photodynamic therapy (PDT) mediated by different combinations of aluminium phthalocyanine chloride (AlPcCl) nanoemulsion [PS] and hydralazine (HLZ) on the proliferation of *C. neoformans* a) H99, b) *hda*1Δ, c) *hos*2Δ, d) *hda*1Δ/*hos2*Δ and e) *hda*1Δ + HDA1 strains. The Y-axis indicates the absorbance value at 600 nm after 72 h. dark (—), light (▬) (*) <0.01 and (**) <0.001 correspond to statistically significant difference from the control, determined by two-way ANOVA and multiple comparisons by Dunnett's test. 50% PS indicates 6.25 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 3.13 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ, 75% PS indicates 9.38 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 4.69 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ and 100% PS indicates 12.5 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 6.25 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ.

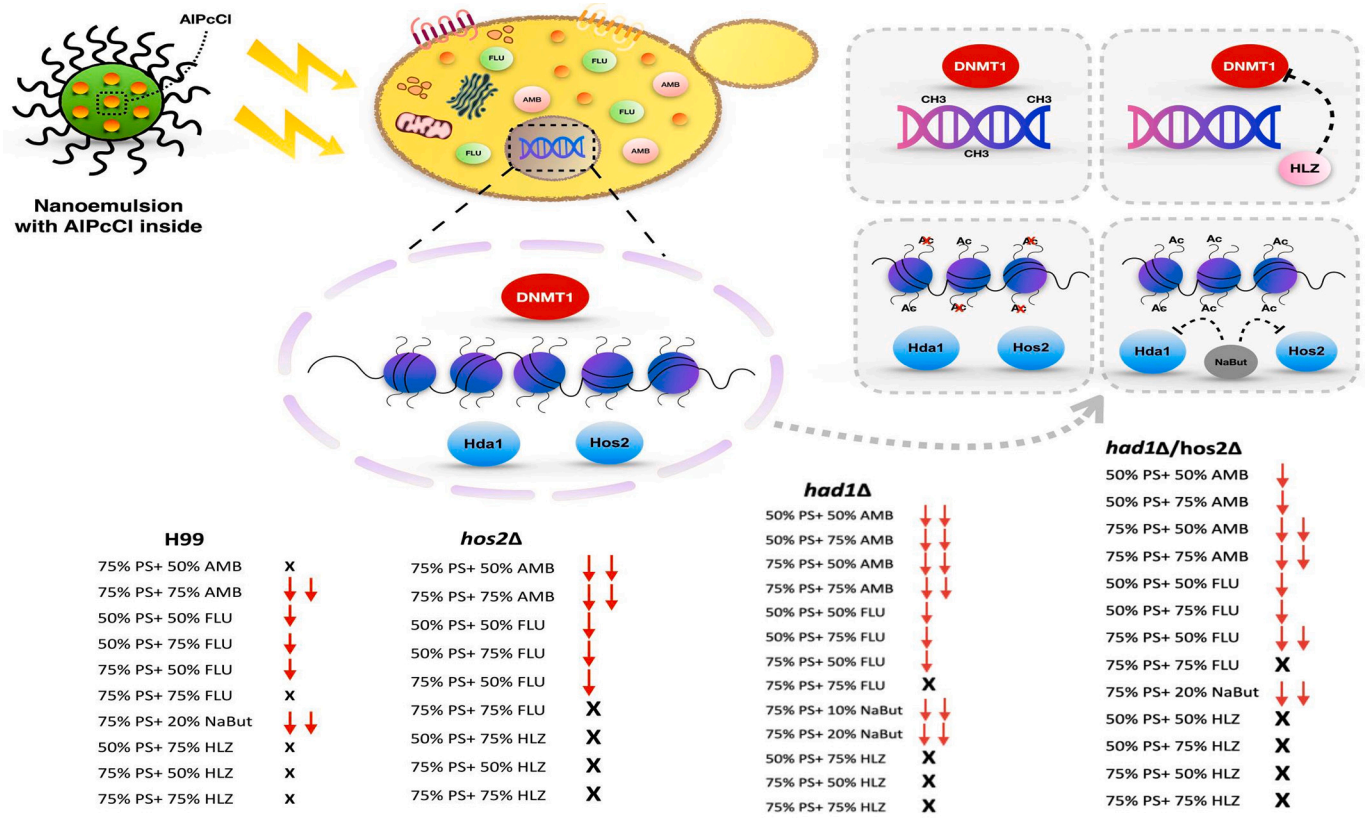

Fig. 7. Effect of Photodynamic interaction on *C. neoformans*. The scheme highlights the efficacy of photodynamic therapy (PDT) employing aluminium-phthalocyanine chloride nanoemulsion (AlPcCl) as photosensitizer agent, in different combinations with antifungals: amphotericin B (AMB), fluconazole (FLU), and Epidrugs: sodium butyrate (NaBut) or hydralazine (HLZ) against the *C. neoformans* H99 wild-type strain and HDAC mutants (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ). The aforementioned drugs increased the lethality of PDT in all the strains. The combinations either abolished (X) or reduced (↓ for * <0.01 and ↓↓ for ** <0.001) the yeast growth. The deletion of the *hda1* or both *hda1* and *hos2* gene increased the efficacy of PDT in comparison to the wild-type strain H99, suggesting a possible link between HDAC genes in the response to the oxidative stress induced by PDT.

collaborators [49] reported a cutaneous cryptococcosis case in a kidney transplant recipient. This patient received liposomal amphotericin and flucytosine for 6 weeks and then oral fluconazole was prescribed as maintenance therapy for all his life.

We believe that PDT following the exposure to AMB, FLU, or HLZ, a drug that is employed for human blood pressure control for decades [50], could be proposed as an alternative treatment regimen for the cutaneous manifestations of *Cryptococcus* infections.

The *hda*1Δ and *hda*1Δ/*hos*2Δ mutants were more susceptible to PDT alone and in combination with AMB and NaBut or HLZ. This may reflect the metabolic differences provoked by the severe impairment of the HDAC machinery, particularly in the double mutant. It is reasonable to speculate that HDAC genes of *Cryptococcus* could also be related to the response to the PDT-induced oxidative stress. Tribus and colleagues revealed that drugs such as paraquat and menadione, which generate oxidative stresses, led to remarkable growth reduction in the *A. nidulans hdaA*Δ mutant. Hydrogen peroxide was lethal to the *hdaA*Δ and *hdaA*Δ/*hosB*Δ mutants, but not to *hosB*Δ [51]. Akiyama and colleagues also reported growth reduction with H_2O_2, paraquat, and menadione for the *Penicliium brasilianum clr3* (*HDA1* equivalent) null mutant [52]. These studies give support to our assumption that *C. neoformans* HDAC mutants are more susceptible to the oxidative stress produced by PDT.

This study is a pathfinder in the investigation of the combined action of antifungal drugs, epigenetic modulators, and PDT in opportunistic fungal pathogens; a challenge to public health in regard to immunocompromised individuals. Our data contribute both to the elucidation of biological processes and to the research on alternative treatments for superficial mycoses.

5. Conclusions

Our results clearly demonstrated that epigenetic changes (induced by chemical inhibitors or by HDAC genes deletion) provoke increased drug susceptibility in *Cryptococcus*. There seems to be an interplay between the HADC activity and the response to PDT-induced oxidative stress, as revealed by the study with gene deletion strains, graphically summarized in Fig. 7. The combination of antifungal drugs, epigenetic modulators and PDT resulted in synergic effects in H99 and HDAC *C. neoformans* strains growth, paving the way to the investigation of alternative treatments for superficial *Cryptococcus* infection in animal models.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2021.112131.

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Declaration of Competing Interest

No potential conflicts of interest were disclosed.

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Table S1: Fractional inhibitory concentration index (FICI) values for the combinations of antifungal drugs and epigenetic modulators for the *C. neoformans* H99, T1 and 89-610 strains and for *C. gattii* NIH198. a) combinations of AMB or FLU with NaBut, TSA, HLZ or 5-AzadC b) combinations of SMX with NaBut, TSA, HLZ or 5-AzadC.

a

	FICI for drug combinations							
Strains	AMB× NaBut	AMB× TSA	AMB× HLZ	AMB× 5-AzadC	FLU× NaBut	FLU× TSA	FLU× HLZ	FLU× 5-zadC
***C. neoformans* H99**	1.09 (I)	0.96 (I)	0.75 (I)	1.01 (I)	0.97 (I)	1.56 (I)	0.62 (I)	1.03 (I)
***C. neoformans* T1**	1.03 (I)	1.17 (I)	1.03 (I)	1.01 (I)	1.34 (I)	1.87 (I)	0.55 (I)	0.75 (I)
***C. neoformans* 89-610**	1.19 (I)	1.21 (I)	0.93 (I)	0.62 (I)	1.47 (I)	1.50 (I)	0.55 (I)	1.16 (I)
***C.gattii* NIH198**	1.06 (I)	1.67 (I)	1.50 (I)	1.00 (I)	1.03 (I)	1.28 (I)	1.06 (I)	0.55 (I)

b

	FICI for drug combinations			
	SMX× NaBut	SMX× TSA	SMX× HLZ	SMX× 5-AzadC
***C. neoformans* H99**	0.65 (I)	0.55 (I)	0.65 (I)	2.15 (I)
***C. neoformans* T1**	1.00 (I)	1.25 (I)	1.83 (I)	1.83 (I)
***C. neoformans* 89610**	0.75 (I)	1.21 (I)	0.93 (I)	0.62 (I)
***C.gattii* NIH198**	1.07 (I)	0.63 (I)	1.07 (I)	0.75 (I)

FICI: Fractional inhibitory concentration index, I: Indifference, S: Synergism, AMB: Amphotericin B, FLU: Fluconazole, SMX: Sulfamethoxazole, NaBut: Sodium butyrate, TSA: Trichostatin A, HLZ: Hydralazine and 5-AzadC: 5-aza-2′-deoxycytidine.

Table S2: Fractional inhibitory concentration index (FICI) values of combinations of antifungal drugs and epigenetic modulators for *C. neoformans* H99 and histone deacetylase mutant strains a) combinations of AMB or FLU with NaBut, TSA, HLZ or 5-AzadC b) combinations of SMX with NaBut, TSA, HLZ or 5-AzadC.

a

	FICI for drug combinations							
	AMB× NaBut	AMB× TSA	AMB× HLZ	AMB× 5-AzadC	FLU× NaBut	FLU× TSA	FLU× HLZ	FLU× 5-AzadC
H99	0.75 (I)	1.03 (I)	1.12 (I)	0.53 (I)	1.50 (I)	1.01 (I)	0.62 (I)	1.12 (I)
***hda1*Δ**	0.62 (I)	1.01 (I)	0.62 (I)	0.62 (I)	1.12 (I)	2.03 (I)	0.75 (I)	0.31 (s)
***hos2*Δ**	0.62 (I)	0.62 (I)	0.75 (I)	1.03 (I)	2.00 (I)	1.01 (I)	0.75 (I)	0.62 (I)
***hda1*Δ/*hos2*Δ**	0.62 (I)	0.53 (I)	0.73 (I)	0.75 (I)	0.37 (S)	0.50 (s)	0.75 (I)	0.75 (I)
hda1*Δ +*HDA1	1.50 (I)	0.75 (I)	0.51 (I)	1.00 (I)	1.00 (I)	2.01 (I)	0.62 (I)	1.07 (I)

b

	FICI for drug combinations			
	SMX× NaBut	SMX× TSA	SMX× HLZ	SMX× 5-AzadC
H99	1.50 (I)	0.37 (s)	0.75 (I)	0.75 (I)
***hda1*Δ**	2.00 (I)	0.18 (s)	1.00 (I)	1.25 (I)
***hos2*Δ**	2.00 (I)	0.75 (I)	1.00 (I)	0.75 (I)
***hda1*Δ/*hos2*Δ**	0.25 (s)	0.12 (s)	0.37 (s)	1.25 (I)
***hda*1Δ+HDA1**	1.00 (I)	1.00 (I)	1.00 (I)	1.25 (I)

FICI: Fractional inhibitory concentration index, I: Indifference, S: Synergism, AMB: Amphotericin B, FLU: Fluconazole, SMX: Sulfamethoxazole, NaBut: Sodium butyrate, TSA: Trichostatin A, HLZ: Hydralazine and 5-AzadC: 5-aza-2′-deoxycytidine.

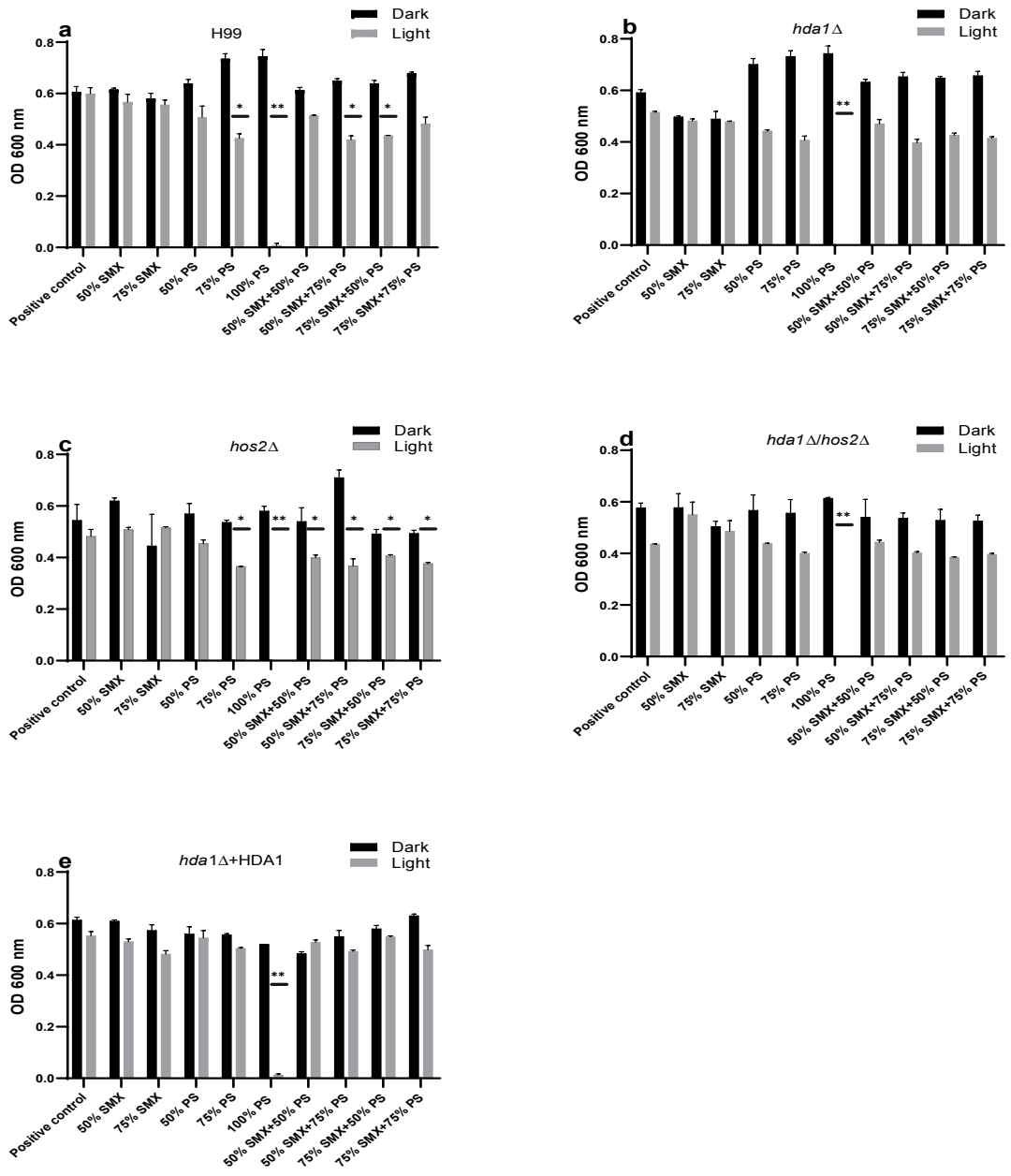

Fig. S1. Bar graph representation of the effect of photodynamic therapy (PDT) mediated by different combinations of nanoemulsion aluminium phthalocyanine chloride (AlPcCl) [PS] and sulfamethoxazole (SMX) on the proliferation of *C. neoformans* a) H99, b) *hda*1Δ, c) *hos*2Δ, d) *hda*1Δ/*hos2*Δ and e) *hda*1Δ + HDA1 strains. The Y-axis indicates the absorbance value at 600 nm after 72 h. dark (▬), light (▬) (*) <0.01 and (**) <0.001 correspond to statistically significant difference from the control, determined by two-way ANOVA and multiple comparisons by Dunnett's test. 50% PS indicates 6.25 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 3.13 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ, 75% PS indicates 9.38 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 4.69 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ and 100% PS indicates 12.5 nM AlPcCl for H99, *hos*2Δ and *hda*1Δ + HDA1 and 6.25 nM for *hda*1Δ and *hda*1Δ/*hos2*Δ

```
library(ggplot2)
library(forcats)
library(RColorBrewer)
library(readxl)


##Gradient green color
col.pal <- c('#ffffff','#e5f5f9','#ccece6','#99d8c9','#66c2a4','#41ae76','#238b45','#006d2c','#00441b')

Synergism <- read_excel('Synergismdata.xlsx')



## Tile plot
ggplot(Synergism, aes(x=factor(Drugcomb, level = c("AMB×NaBut","AMB×HLZ","AMB×TSA","AMB×
5-AzadC ","FLU×NaBut","FLU×HLZ","FLU×TSA","FLU× 5-
AzadC","SMX×NaBut","SMX×HLZ","SMX×TSA","SMX× 5-AzadC ")),
                y=factor(Strain, level = c("hda1Δ+HDA1","hda1Δ/hos2Δ","hos2Δ","hda1Δ","H99")),
fill=FICvalues)) +
  geom_raster() +
  theme(axis.text.x = element_text(angle = 90, hjust = 1, size = 11), axis.text.y = element_text(size =
11), plot.title = element_text(hjust=0.5)) +
  guides(fill=guide_legend(title="FICI values")) +
  xlab("Drug combinations") +
  ylab("Strains") +
  scale_fill_gradientn(colours=rev(col.pal), breaks=c(0.12,0.25,0.5,1.0,1.5))+
  coord_fixed(ratio=1)
```

CHAPTER II

Antifungal potential of new actinobacteria isolates obtained from the rhizosphere of Indian Traditional Medicine against *Cryptococcus*

2 Introduction

2.1 Actinobacteria

Drug-resistant microbes causing infections, including fungi, are becoming a major worldwide problem. There is a need to find alternative antimicrobial agents to combat them. Actinobacteria are an important source of bioactive substances for the pharmaceutical industry. Actinobacteria, mostly *Streptomyces,* produce two thirds of the available antibiotics (Mahajan and Balachandran, 2014; Liu et al., 2018).

The phylum Actinobacteria consists of Gram-positive bacteria presenting a high DNA guanine + cytosine content (from 51% in *Corynebacterium* species. to more than 70% in genera *Streptomyces* and *Frankia*; VENTURA et al., 2007). Actinobacteria represent a separate but large taxonomic component of the Bacteria domain, and present ubiquitous distribution (reviewed by BARKA et al., 2016). Most of the actinobacteria are free-living organisms, in terrestrial or aquatic ecosystems, including the marine environment (MACAGNAN et al., 2006). Animal or plant pathogens (species of *Tropheryma*, *Nocardia*, *Corynebacterium*, *Propionibacterium* and *Mycobacterium*), gastrointestinal commensals (*Bifidobacterium* sp.) and plant commensals were also reported (VENTURA et al., 2007; BARKA et al., 2016).

Actinobacteria are abundant in the soil, where they correspond to one of the most abundant phyla of the microbial population, and can be found from the surface to more than 2 meters deep (GOODFELLOW; WILLIAMS, 1983).

Initially, actinobacteria were described as a transitional form between bacteria and fungi, due to the formation of mycelium by many species. Many of the mycelium-forming actinobacteria undergo hyphae differentiation, leading to reproductive spore formation. Nonetheless, the comparison to fungi is inaccurate: actinobacteria present thin cells, with genetic material contained in a prokaryotic nucleoid; the cell wall is composed of

peptidoglycans and they are susceptible to most of the antibacterial agents. Based on 16S rDNA sequencing, the phylum Actinobacteria previously included 5 subclasses, 6 orders and 14 suborders (LUDWIG et al., 2012). New genetic markers, such as the *rpoB* and *ssgB* genes have also been employed to classify actinobacteria (GIRARD et al., 2013).

Recently, using phylogeny of 16S rDNA sequences, Salam and colleagues (2020) updated the taxonomical classification of actinobacteria into 6 classes, 46 orders and 79 families, including 16 new orders and 10 new families.

Most of the actinobacteria life cycle corresponds to the spores state, mainly due to the lack of nutrients in the environment (MAYFIELD et al., 1972). Factors such as soil pH, temperature and moisture also influence the growth of these organisms. *Streptomyces* spores are normally resistant to desiccation (CHATER et al., 2010).

While many bacteria are dispersed, adhered to soil particles, certain bacteria interact directly with plants roots. The term “rhizosphere” describes the soil region under the action and influence of a plant root. Therefore, it represents the region where soil and biota interact (LYNCH; DE LEIJ, 2012). The concentration of bacteria per gram of soil in the rhizosphere is, in general, much higher than in the rest of the soil (GLICK, 1995).

Actinobacteria in rhizosphere soil can influence the growth of symbiotic plants by preventing the harmful effects of other microorganisms in the environment. Colonization and the synthesis of secondary metabolites products prevent the proliferation of pathogens (FENTON et al., 1992). Actinobacteria can also promote plant growth by supplying metabolic products, such as phytohormones, or by facilitating the obtainment of soil nutrients, as in nitrogen fixation (GLICK, 1995). On the other hand, root exudates stimulate actinobacteria growth.

2.2 Morphology

Actinobacteria covers genera of the most varied morphologies. There are coccoid organisms, such as *Micrococcus*, or cocobacillary organisms, such as Arthrobacter, which, as members of the genus *Corynebacterium*, can reproduce by binary fission. Others, such as *Nocardia* and *Mycobacterium* species, show filamentous growth with subsequent fragmentation. There are also organisms, such as *Actinoplanes* and *Streptomyces*, that present filamentous growth with the formation of permanent branched highly differentiated mycelium (CHATER; HOPWOOD, 1993). The formation of reproductive spores from aerial mycelium in *Streptomyces* species (BARKA et al., 2016), and of persistent non-replicative structures in some *Mycobacterium* (VENTURA et al., 2007) is well described.

2.3 The *Streptomyces* genus

Streptomyces are the most abundant actinobacteria in the soil (95% of Actinomycetales isolates). They are capable of degrading polysaccharides, such as cellulose, chitin, xylan due to the production of hydrolases. Numerous secondary metabolites employed in medicine and industry were also characterized from *Streptomyces* (BARKA et al., 2016).

Since the report of the genomic sequence of the model species *Streptomyces coelicolor* (BENTLEY et al., 2002), the genome of several species of this genus has been sequenced.

The life cycle of *Streptomyces* species begins with the germination of a spore, when the environment presents favourable conditions (Figure 7). From the spore germination, vegetative hyphae grow to form the intricately branched mycelium which is characteristic of the genus (CHATER, 1972). Streptomycetes mycelia elongate through extension and branching of the hyphae tips. Cell division does not lead to cell fission, but to the formation of irregularly spaced cross-walls through the hyphae (WILDERMUTH; HOPWOOD, 1970). Thus, *Streptomyces* presents multicellular mycelium, with interconnected compartments containing numerous copies of the genetic material (CLAESSEN et al., 2014).

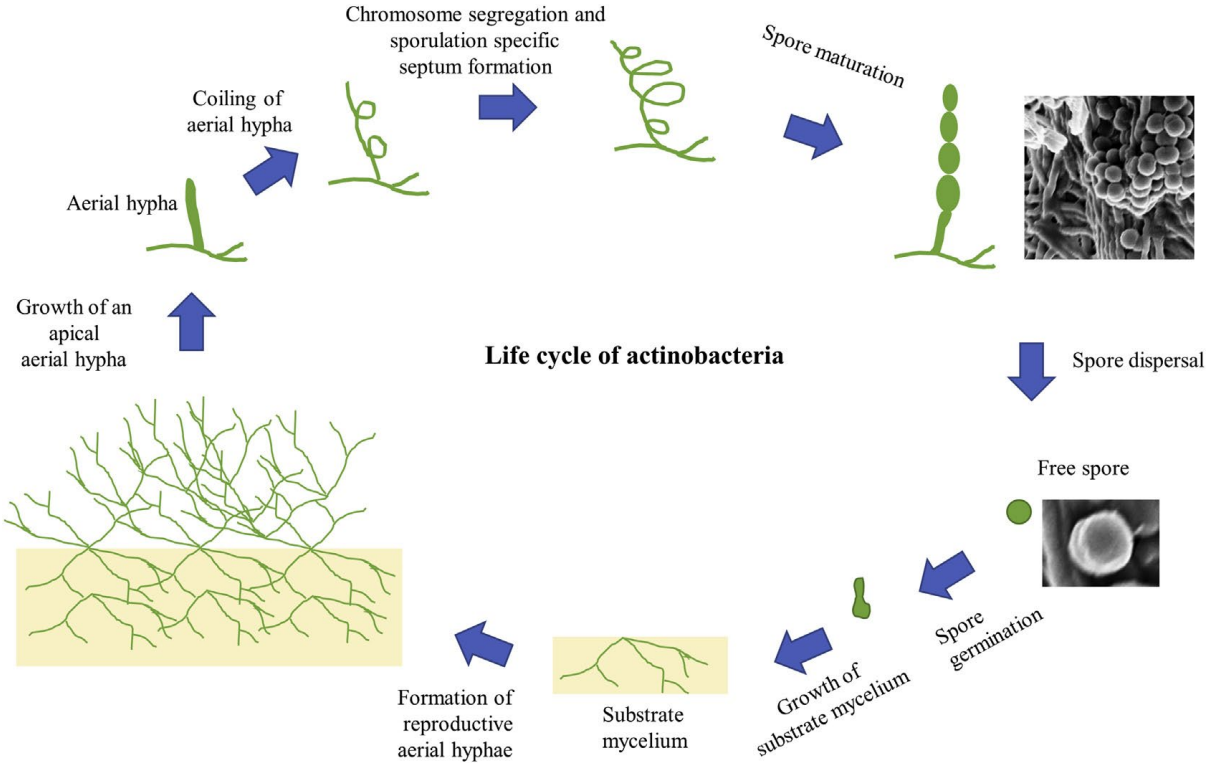

Figure 5: Illustrative representation of the life cycle of Actinobacteria presenting sporulation, maturation and germination of spores. Adapted from HAZARIKA; THAKUR, (2020).

Under certain conditions, the vegetative mycelium of *Streptomyces* undergoes cell differentiation, giving rise to spore chains, which are responsible for the characteristic velvety surface the colony presents on solid substrates. After dispersal, the spores give rise to a new cycle (CLAESSEN et al., 2014).

It is believed that the production and release of antibiotics through the life cycle allows the competition for colonization, nutrient uptake and growth (CHATER; HOPWOOD, 1993). The production of secondary metabolites in *Streptomyces* is generally associated with cell differentiation and with the growth of spore-forming aerial mycelium (BIBB, 2005).

2.4 Antimicrobial bioactive metabolite from actinobacteria

Actinomycin was the first antibiotic isolated from actinobacteria (WAKSMAN; WOODRUFF, 1940). In the same decade, WAKSMAN; WOODRUFF, (1942a, 1942b) isolated streptothricin from *Streptomyces lavendulae*. Streptomycin, isolated from

Streptomyces griseus (SCHATZ; BUGLE; WAKSMAN, 1944; SCHATZ; WAKSMAN, 1944), was the first antibiotic obtained from the genus *Streptomyces* to be applied in therapeutics (CHATER et al., 2010).

The phylum actinobacteria produce approximately 70% of antibiotics known today. The *Streptomyces* genus is responsible for half of the compounds currently in clinical trials (AVALOS et al., 2020). The production capacity between species varies. That is, while some species of *Streptomyces* produce only one antibiotic, others are capable of producing a series of compounds of different classes (BARKA et al., 2016).

Al-Dhabi and colleagues (2019) described a thermophilic *Streptomyces* isolate from the Saudi Arabian desert. This isolate inhibited the growth of *C. neoformans*, *Trichophyton mentagrophytes*, *C. albicans* and *Aspergillus niger* by producing benzene acetic acid and methoxy-, 2-phenylethyl ester.

AVALOS and co-workers (2020) discussed the production of volatile compounds by streptomycetes, which are dispersed in the environment and interfere with the growth of other microorganisms at a distance. In nature, such volatile compounds can promote or inhibit growth, modulate drug resistance or quorum sensing. In this way, they influence the phenotype and behaviour of microorganisms around them (AUDRAIN et al., 2015). Volatile compounds are synthesized and secreted with low energy investment and can inhibit the growth of Gram-positive and Gram-negative bacteria over long distances. They correspond to ammonia molecules of high vapor pressure and low molecular weight which diffuse easily through air, water or soil. The authors also suggested that volatile compounds released by *Streptomyces* aid in the solubility and diffusion of more complex antimicrobial secondary metabolites. In this way, the small ammonia antibiotic molecules could sensitize competing bacteria, prior to the synthesis of complex antibiotics, such as polyketides.

2.5 Traditional medicine plants

The employment of plant parts - mostly leaves, roots and stems – for the treatment of ailments represent one of the most ancient practices of mankind. Only much later, the fractionation techniques allowed the obtainment of crude extracts, powders and partially purified compounds.

In countries like India and China, herbal medicine has been traditionally used for thousands of years for the treatment of skin diseases, infections, aches, as anti-parasitic and also in cosmetology. These days, herbal practice represent the only available treatment for the underprivileged society layers, especially in rural and remote areas. On the other hand, in these countries, herbal therapy has achieved a considerable degree of scientific knowledge and sophistication, with millionaire brands on the market (QIN et al., 2009; ZHAO et al., 2012).

As pointed out by TANDON (2017), the World Health Organization has been supporting phytomedicines or herbal drugs as an effective alternative therapy.

Considerable scientific advances have been achieved on botanical biodiversity, on the discovery of novel therapeutic properties of known plants, extraction methods, physical-chemical characterization of active principles and on the development of new formulations. On the other hand, not much information on products derived from actinobacteria associated with the medicinal plants' rhizosphere is available.

In this work, in collaboration with the Department of Microbiology of the Maharshi Dayanand University (Rohtak, India), we aimed to isolate actinobacteria from the rhizosphere of Indian traditional medicine plants and to assess their potential antifungal activity

2.6 Medicinal plants employed in this work

2.6.1 *Helianthus annuus*

H. annuus, common sunflower, is a tall and fast-growing annual plant (Figure 8). Seed preparations are used for the treatment of colds, cough, bronchial and pulmonary infections. A tincture prepared from seeds and wine is useful for the treatment of fever (BASHIR et al., 2015).

A protein obtained from *H. annuus* completely inhibited the growth of the plant pathogen *Fusarium solani* f. sp. *eumartii*. It also abolished spores germination at the concentration of 40 μg mL^{-1} (REGENTE; DE LA CANAL, 2001).

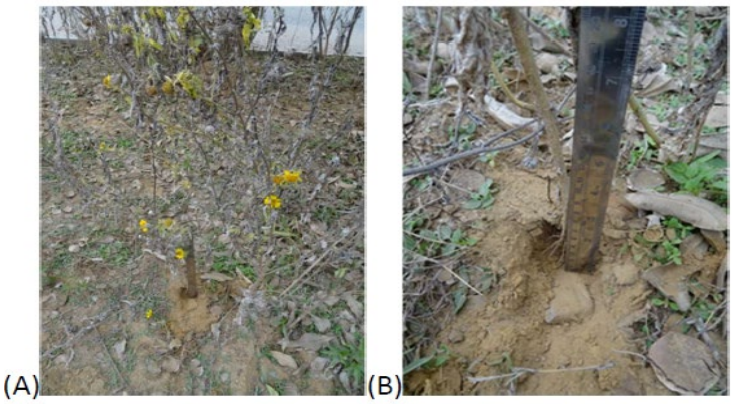

Figure 6: *H. annuus* flowering bush (A) and detail of the area from which the rhizosphere soil sample was collected (B).

2.6.2 *Pongamia pinnata*

P. pinnata (common names “karanj” or “papdi”) is a medium-sized and fast-growing, evergreen shrub (Figure 9). Preparations from the roots are effective for treating gonorrhoea, vaginal and skin affections, ulcers and also for cleaning gums and teeth (MUTHU et al., 2006). In southern India, tribal populations use this plant for the treatment of wounds (AL MUQARRABUN et al., 2013).

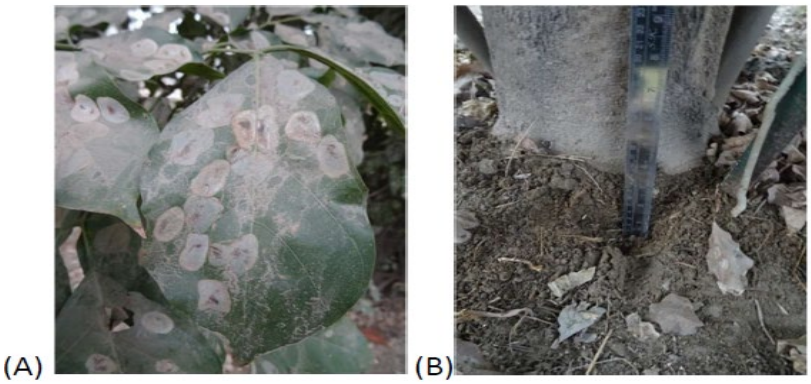

Figure 7: *P. pinnata* leaves (A) and detail of the area from which the rhizosphere soil sample was collected (B).

2.6.3 *Ziziphus mauritiana*

Z. mauritiana (common names “ber” or “badari”) is a spiny, evergreen shrub native from India (Figure 10). It produces a drought, hard fruit; it is found in the wild and as a cultivated species. The seeds are given to stop nausea and vomiting and for the relief of abdominal pain during pregnancy. In the state of Bihar, India, the seeds are used to treat diarrhoea. The fruit is also referred in the popular pharmacopoeia as an oral contraceptive. A decoction of the bark is also used to treat diarrhoea and dysentery (GOYAL; NAGORI; SASMAL, 2012)

Figure 8: *Z. mauritiana* branches and fruits (A) and detail of the area from which the rhizosphere soil sample was collected (B)

2.7 JUSTIFICATION

From the basic and medical research point of view, the search for alternative drugs and therapeutic regimens for pathogenic microorganisms of high prevalence in fungal infections is relevant. The increasing incidence of resistance to conventional antifungal drugs anticipate the importance of studies such as proposed here.

The screening of actinobacteria isolates, from unexplored sources, capable of producing new antifungal compounds is compelling, aiming the control of *Cryptococcus* growth.

2.8 Objectives

a) To isolate actinomycetes from rhizosphere soils of Indian traditional plants and to screen them for the ability to produce antimicrobial substances against *Cryptococcus*

b) To determine the minimum inhibitory concentrations (MICs) of the crude extract from the obtained actinobacteria against *C. neoformans* H99 (reference strain), T1 and 89-610 (fluconazole-resistant strains), *C. gattii* NIH198, and the HDAC null mutant strains (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ, *hda1*Δ+HDA1).

Cryptococcus sp. growth inhibition activity of new actinobacteria isolates obtained from the rhizosphere of Indian Traditional Medicine plants

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Keywords: Actinobacteria, Indian Traditional Medicine plants, antifungal activity *Cryptococcus,* Amazonian iron ore mining area fungi.

Abstract

Actinobacteria of the genus *Streptomyces* are part of the soil microbiota. Nonetheless, studies on actinobacteria isolated from the rhizosphere of traditional medicine plants are scarce. In the present study, four actinobacteria of the genus *Streptomyces* (*S. gancidicus*, *S. griseoruber* and *S. aureus*) were isolated from the rhizosphere of the Indian Traditional Medicine plants *Helianthus annuus*, *Pongami pinnata* and *Ziziphus mauritiana* and phylogenetically analysed. The antifungal activity of the crude extract of the *Streptomyces* isolates was evaluated against different *Cryptococcus neoformans* strains, *C. neoformans* histone deacetylase genes null mutants and *C. gattii* NIH198 by the microdilution method. Our results showed that the crude extract of *S. griseoruber* Pp1 isolate inhibited the growth of the fluconazole-resistant *C. neoformans* T1 strain more intensely than the H99 reference strain. The HDAC gene deletion mutants were more susceptible to the crude extract of *S. aureus* isolate UzM in comparison to H99, especially the *hda1*Δ and *hda1*Δ/*hos2*Δ strains. Our results indicate that these actinobacteria isolates shall be further studied as sources of new antifungal compounds which could help to circumvent the escalating problem of drug resistance in *Cryptococcus*.

Key words: *Cryptococcus* spp., drug resistance, actinobacteria, traditional medicine plants, antifungal activity.

Introduction

Cryptococcus is an opportunistic fungal yeast pathogen that causes cryptococcosis. It is estimated that more than 223,000 cases of cryptococcal meningitis occur each year in the world (RAJASINGHAM et al., 2017). *Cryptococcus neoformans* infects mainly immunocompromised patients and *Cryptococcus gattii* infects immunocompetent hosts as well (CHAYAKULKEEREE; PERFECT, 2006; RAJASINGHAM et al., 2017). The extensive employment of antifungals, such as amphotericin B and fluconazole, is provoking an increasing selection of drug resistant *Cryptococcus* isolates in the world. Genomic/phenotypic plasticity is generated by aneuploidy, epigenetic fluctuation, transposons movement and mutations in the genes which are target for the conventional antifungal drugs. These adaptations allow *Cryptococcus* to develop resilience to antifungal assaults (STONE et al., 2019; BILLMYRE et al., 2020; GUSA et al., 2020). Due to limitations for the treatment of *Cryptococcus* infections, there is an urgent need for alternative treatment regimens.

Actinobacteria have attracted attention for many years due to the capability to produce new antimicrobial compounds. Nonetheless, the literature on actinobacteria isolates capable of inhibiting *Cryptococcus* spp. is very limited (e.g. VARTAK et al., 2014a; OUARGLI et al., 2015; NITHYA et al., 2018).

Medicinal plants seem to represent promising sources of rare actinobacteria. Studies on actinobacteria isolated from Indian medicine plants described antimicrobial activities against pathogenic bacteria (GOHAIN et al., 2019) and plant pathogen fungi (GOHAIN et al., 2019; PASSARI et al., 2015). However, there are not many studies on the antifungal potential against human pathogens.

In face of the above, our goal in the present study was to isolate actinobacteria from the rhizosphere of *Helianthus annuus* (common wild sunflower), *Pongamia pinnata* (common names “karanj”) and *Ziziphus mauritiana* (common names “ber”), from the rural area of

Rohtak, State of Haryana, India, and to evaluate the potential of growth inhibition against *Cryptococcus neoformans* strains, *C. neoformans* histone deacetylase genes null mutants, *C. gattii* NIH198.

Our results demonstrated the antifungal potential of actinobacteria isolated from Indian medicine plants to fluconazole-resistant *Cryptococcus* strains. The actinobacteria isolates also inhibited the growth of histone deacetylase (HDAC) gene deletion null mutants, which suggest a link between actinobacteria-derived metabolites and the *Cryptococcus* histone acetylation/deacetylation processes.

Materials and methods

Soil sampling and actinobacteria isolation (Marcio J. Poças-Fonseca, personal communication)

Soil samples were collected from about 5 centimetres depth diggings in the rhizosphere of *Helianthus annuus* (Ha), *Pongami pinnata* (Pp) and *Ziziphus mauritiana* (Zm) plants located in the outskirts of the Rohtak district (Haryana, India). After collection, soil samples were air-dried for 24 h, ground and 1 g of each sample was transferred to 9 mL 0.9% sterile NaCl. The mixtures were vigorously shaken for 2 min and soil particles were allowed to settle down for approximately 30 min at room temperature. Five 1:10 serial dilutions were made for each suspension and 200 µL of the 10^{-4} and 10^{-5} was inoculated on Asparagine Glycerol Agar (AGA) (1% glycerol, 0.1% L-asparagine, 0.1% dibasic potassium phosphate, 0.01% trace salt solution for actinomycete, 1.5% agar) plates. The plates were incubated at 28 ºC for two weeks and observed daily.

Morphologically distinct colonies with an actinomycete-like appearance were seeded to single colonies onto AGA medium plates containing nalidixic acid (20 µg mL-1). The purity

of actinobacteria cultures was confirmed under the light microscope Carl Zeiss Axioskop 20 EL-Einsatz 451487 Binocular Transmitted Light Microscope (Oberkochen, Germany) and preserved at −80°C in 30% (v/v) of glycerol. The colonies were named using the code "Ha" for *H. annuus*, "Pp" for *P. pinnata* and "Uz" for *Z. mauritiana*.

DNA extraction and PCR amplification of the ITS region of the obtained actinobacteria isolates

Pure isolates of actinobacteria were inoculated in 3 mL of NDB (0.3% beef extract, 0.5% peptone, 1% dextrose) at 28 °C for 3 to 5 days and 150 rpm. Mycelium was collected and washed three times with Tris-EDTA (TE) buffer (1 M Tris-Cl, 0.5 M EDTA, pH 8.0). Then TE, CTAB-NaCl (2%), NaCl (1.3 M), lysozyme (0.2 mg mL^{-1}), proteinase K (0.2 mg mL^{-1}) and sodium dodecyl sulphate (SDS) (0.35%) were added to the washed mycelium. The hyphae were macerated and vortexed vigorously in the presence of glass beads to lyse the cells. Mixtures were incubated at 37 °C for 1 h and then 65 °C for 15 min and centrifuged. The aqueous phase was then treated with RNase A (10 µg mL^{-1}) for 45 min at 37 °C. The samples were deproteinated twice with a phenol: chloroform: isoamyl alcohol (25: 24: 1) solution, followed by extraction with a chloroform: isoamyl alcohol (24: 1) solution. For DNA precipitation, sodium acetate (0.3 M) and isopropanol (0.6 v/v) were added and the samples were centrifuged at 13,000 rpm for 10 min. The DNA pellet was washed twice with chilled 70% ethanol and resuspended in sterile MilliQ water. Extracted DNA was kept at -20 °C before further analyses.

The genomic DNA was amplified using the primers specific to actinobacteria 16S gene (SC-Act-235aS20: 5'-CGCGGCCTATCAGCTTGTTG and SC-Act-878aA19: 5'-CCGTACTCCCCAGGCGGGG) (CHÄFER; JÄCKEL; KÄMPFER, 2010). All PCR amplification reactions were performed in the final volume of 25 µL, containing 1× Taq buffer, 2 mM $MgCl_2$, 800 µM dNTP mix, 0.4 pmol of each primer, 1.2 U Taq DNA polymerase

(Invitrogen) and 1 µL of DNA as a template. An initial denaturation at 95 °C for 3 mins was followed by 35 cycles of 95 °C for 30 s; 70 °C for 60 s and 72 °C for 60 s with final extension at 72 °C for 10 min. The PCR products were analysed by electrophoresis on a 1.2% agarose gel containing ethidium bromide (0.5µg L^{-1}).

The PCR products were then treated with the SAP (0.18 U μL^{-1}) and EXOI (0.2 U μL^{-1}) enzymes at 37 °C for 1 h 30 min, followed by 80 °C for 20 min and 4 ° C for 20 min. The samples were sent for sequencing in the final volume of 7 µL containing 3 µL of primer (10 µM) and 5-20 ng of DNA at the DNA Sequencing Laboratory at the Catholic University of Brasilia (UCB), using the Genetic Analyzer 3130 (Applied Biosystems) sequencer.

Phylogenetic tree analysis of the actinobacteria isolates

Similarity search for the DNA sequences was performed against the NCBI database using BLAST search **(**http://www.ncbi.nlm.nih.gov/**)**. The quality of all sequences was analysed using the DNASTAR Lasergene SeqMan pro (version: 7.1.0) software. Sequences were aligned by the multiple alignment ClustalW program, using the molecular evolutionary genetics analysis package (MEGA version 5.2). Phylogenetic trees were constructed using the same software.

Preparation of crude extracts from the actinobacteria isolates

The actinobacteria isolates were grown on AGA plates for 7 to 10 days at 28 °C for sporulation. Spores were inoculated in seed media for actinomycetes (1% starch, 0.5% glucose, 0.5% yeast extract, 0.5% K_2HPO_4, 0.05% $MgSO_4.7H_2O$) and incubated at 28 °C for 3-5 days at 150 rpm. After incubation, 15 % (V/V) of these cultures were inoculated in a 500 mL Erlenmeyer flask containing 100 mL of fermentation medium for actinomycetes, SCN (1%

starch, 0.03% casein, 0.2% potassium nitrate, 0.2% sodium chloride, 0.2% potassium phosphate dibasic, 0.005% magnesium sulphate, 0.002% calcium carbonate, 0.001% ferrous sulphate heptahydrate). The flasks were incubated at 28 °C for 14 days, 120 rpm, in the dark. After the incubation, cultures were observed under the microscope to check for contamination with other microbes. Extracts were centrifuged to remove the biomass. The supernatant was filtered using a 0.45-micron pore size filter and the pH of each crude extract was measured. The collected biomass of actinobacteria was kept at 50 °C for measurement of the dry biomass. The sterile crude extracts were subjected to lyophilization (Liotop® L101, Liobras, SP, Brazil) and the powder was dissolved in MilliQ water to prepare stock solutions of 80 mg mL^{-1}, which were used for the bioactivity assays.

Anti-cryptococcal activity of the actinobacteria crude extracts

C. neoformans H99, 89–610 and T1 (fluconazole-resistant) strains and *C. gattii* NIH198 were generously provided by Dr Joseph Heitman Laboratory (Duke University Medical Center, Durham-USA). The histone deacetylase null mutants *hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ and the reconstituted *hda1*Δ + HDA1 strains were generated and phenotypically characterized by our group (BRANDÃO et al., 2018).

The minimum inhibitory concentrations (MICs) for the actinobacteria crude extracts were determined against the aforementioned *Cryptococcus* strains according to the NCCLS M27-A2 protocol (WAYNE, 2008). The strains were grown in YPD broth and incubated for 20 h at 30 °C, with shaking. Cells were washed with 1X PBS and the inoculum concentration was adjusted to 4×10^4 cells mL^{-1} in 2X RPMI 1640 media with L-glutamine, without sodium bicarbonate (Sigma Aldrich®, St. Louis, MO, USA), buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). One hundred µL of this solution was added to the

wells containing 100 µL of the 2X concentrated crude extract. Initially, crude extracts were diluted in 2-fold series from 40 mg mL^{-1} to 0.31 mg mL^{-1} according to CLSI protocol. Subsequently, to refine the determination of inhibitory concentrations, crude extracts were diluted in the final concentrations of 10 to 1 mg mL^{-1}. Positive controls without drugs and negative controls without yeast cells were performed. As inhibitory control drug, amphotericin B in MIC concentration was used for each strain, as determined from our previous work (RANJAN et al., 2021).

The MIC assay was also performed with 2X Mueller-Hinton broth (MHB) instead of RPMI, as mentioned above with little modification (Al-Dhabi et al., 2019; Amorim et al., 2020).

The plates were protected from light and incubated at 37 °C for 72 h. After that, endpoints were visually read. MIC was defined as the lowest concentration that completely abolished fungal growth. All the MIC assays were conducted as three independent experiments, with three technical replicates.

Results

Macroscopic and microscopic features of actinobacteria isolates

In a pilot study, we isolated 20 actinobacteria cultures which presented antifungal activity (Poças-Fonseca, data not shown). Four of the most promising ones were selected for further studies.

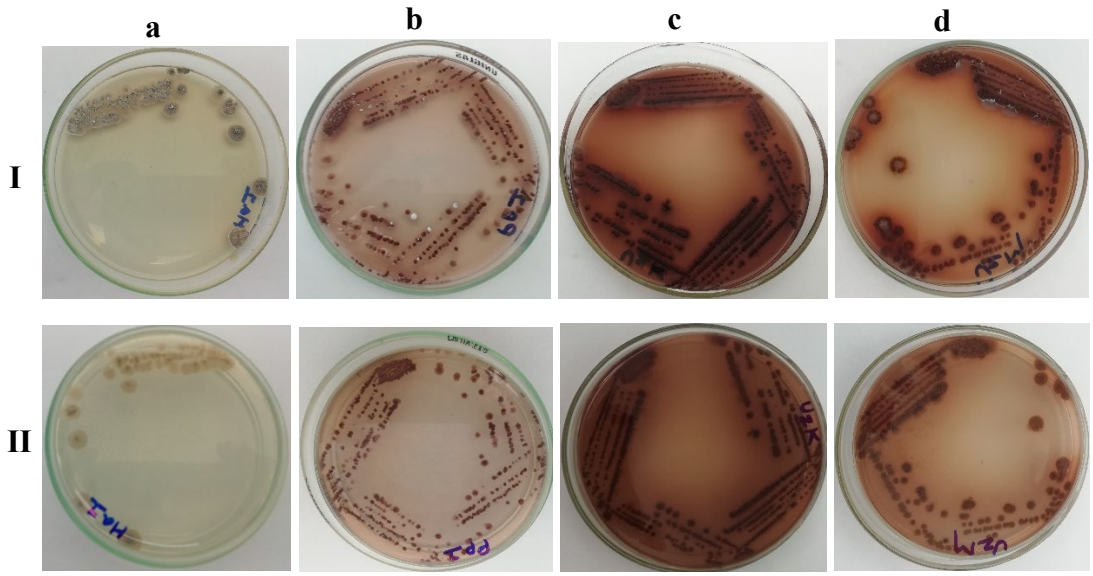

Figure 1: Illustrative picture of pure cultures of the actinobacteria isolates on 90 mm AGA plates. (I) front side and (II) back side. (a) Ha1, b) Pp1, c) UzK and d) UzM.

Once the purity of the four selected isolates was confirmed by optical microscopy of liquid and solid cultures, grown for 7 days, the isolates were inoculated in AGA medium and incubated at 28 °C, for 12 to 14 days. Figure 1 shows the dry aspect of the colonies surface, a common feature for actinobacteria. Colonies also showed adherence to the agar surface, mediated by mycelia growth into the medium.

Supplementary Table S1 summarizes the macroscopic characteristics of the actinobacteria isolates.

The Ha1 isolate did not produce any pigment in AGA media, even after incubation at 28 °C for a long period of time (Figure 1 a)

Isolate Pp1 presented light brown aerial mycelia, produced light brown soluble pigments which could be visually observed as it dispersed into the medium (Figure 1b).

UzK and UzM isolates presented dark brown aerial mycelia and produced dark brown soluble pigments (Figures 1c and 1d).

Microscopically, all the isolates formed branched non-septate hyphae, both in solid and liquid media. The hyphae of the isolates were dense in the centre and more spaced towards the margins of the colonies (Supplementary Figure S1).

Phylogenetic analysis of the actinobacteria isolates

Comparison of the actinobacteria-specific 16s rDNA sequences of the isolates with other sequences previously deposited in the GenBank database indicated that they belong to the genus *Streptomyces*.

All the isolates showed similar percentage identity with more than two *Streptomyces* spp. Through the phylogenetic tree analysis, it was possible to identify the closest evolutionary relationship within the genus *Streptomyces* (Figure 2). The isolate Ha1 was closest to *S. gancidicus*, Pp1 to *S. griseoruber,* and UzK and UzM to *S. aureus*. Ha1 and Pp1 were placed in different clades, while UzK and UzM were positioned in the same clade.

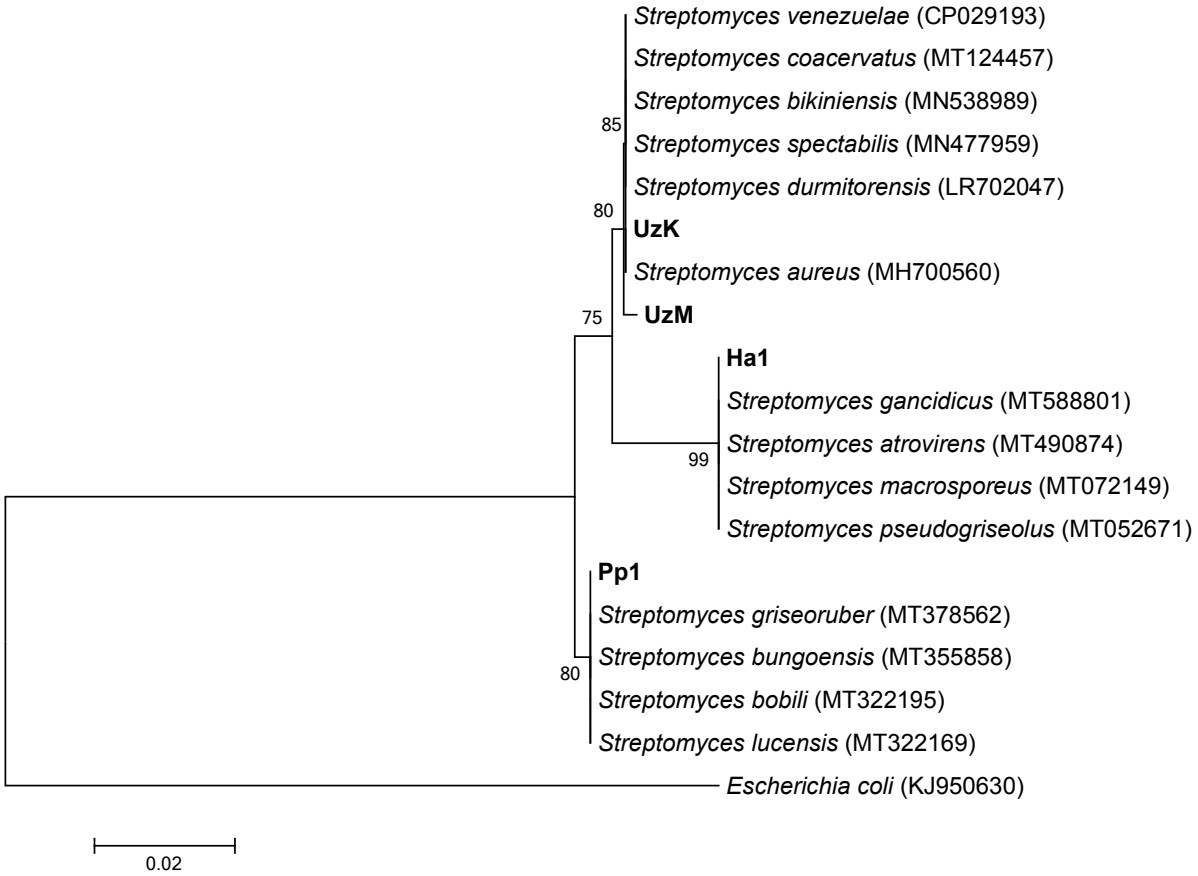

Figure 2 The phylogram showing the position of the actinobacteria isolates with other *Streptomyces* spp. based on the 16s rDNA gene sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Actinobacteria isolates from this study are in bold. Accession numbers are presented in parenthesis

Accession numbers of actinobacteria nucleotide sequences

The 16s rDNA sequences of actinobacteria were submitted to NCBI under the accession numbers: MW965488 for *S. gancidicus* isolate Ha1, MW965489 for *S. griseoruber* isolate Pp1, MW965490 for *S. aureus* isolate UzK and MW965491 for *S. aureus* isolate UzM.

Minimum inhibitory concentrations of the actinobacteria crude extracts against different *C. neoformans* strains and *C. gattii* NIH198

The crude extract of the actinobacteria isolates was collected after 14 days of fermentation in SCN media (Supplementary Figure S1a). *S. gancidicus* isolate Ha1 isolate did not produce any soluble pigment. *S. griseoruber* isolate Pp1 produced soluble black pigment

in SCN, but light brown pigment in AGA. *S. aureus* isolates Uzk and UzM isolates produced diffusible brown pigment.

The pH values of the crude extracts and the dry cell biomass was recorded after fermentation (Supplementary Table S2). The pH value of uninoculated broth was 7.29 and the pH values for the extracts increased to more than 8. The values ranged from pH 8.11 (*S. gancidicus* isolate Ha1) to 8.76 (*S. aureus* isolate UzM).

Even though we were not able to standardize the inoculum, we recorded the dry cell biomass of the isolates after fermentation. *S. gancidicus* isolate Ha1 was the highest (838 mg), followed by *S. aureus* isolate UzM (489). *S. griseoruber* isolate Pp1 isolate produced only 166 mg of dry cell biomass.

The extracts were lyophilized and crude extract powders were resuspended in sterile MilliQ water at the concentration of 80 mg mL^{-1} (Supplementary Figure S1b).

The MICs of the crude extracts against *Cryptococcus* were determined by the microdilution method. There was not much difference in values when RPMI medium was used in the MIC assay (Supplementary Table S3). The *S. gancidicus* isolate Ha1 crude extract did not present MIC for any *Cryptococcus* strain. *S. griseoruber* Pp1 and *S. aureus* isolate UzK extracts presented similar MIC values for all the strains. The HDAC null mutants were more sensitive to the *S. aureus* isolate UzM extract than all the other *C. neoformans* strains.

Next, we performed the MIC experiments with MHB broth instead of RPMI, as suggested by some studies in the literature (AL-DHABI et al., 2019; AMORIM et al., 2020). The results are presented in Table 1.

Table 1: Minimum inhibitory concentrations (MIC) values for the crude extracts obtained from *S. gancidicus* isolate Ha1(SgdHa1), *S. griseoruber* isolate Pp1 (SgrPp1) and *S. aureus* isolates UzK (SaUzK) and UzM (SaUzM) against *C. neoformans* H99, the fluconazole-resistant T1 and 89–610 strains, the histone deacetylase null mutants and against *C. gattii* NIH198.

	Minimum inhibitory concentrations (mg mL^{-1})			
Strains	SgdHa1	SgrPp1	SaUzK	SaUzM
C. neoformans H99	8	>40	7	6.5
C. neoformans T1	4	6	6.5	6.5
C. neoformans 89–610	8	>40	6.5	6.5
*hda1*Δ	8	6	4	2.5
*hos2*Δ	>40	8	4	6.0
*hda1*Δ/*hos2*Δ	6.5	6	4	2
*hda1*Δ+HDA1	8	>40	8	6.5
C. gattii NIH198	>40	>40	6	4

The four different crude extracts inhibited yeasts growth, except for *S. gancidicus* isolate Ha1 against *C. gattii* NIH198, and *S. griseuruber* Pp1 against *C. neoformans* 89–610 and *C. gattii* NIH198.

Table 1 indicates that the fluconazole-resistant *C. neoformans* T1 was two-fold more susceptible (4 mg mL^{-1}) to the *S. gancidicus* isolate Ha1 crude extract in comparison to the H99 reference strain (8 mg mL^{-1}). The histone deacetylase *hda1*Δ/*hos2*Δ double mutant was also more susceptible (6.5 mg mL^{-1}) than H99.

The *S. griseoruber* isolate Pp1 crude extract did not show any inhibition against H99 and 89-610 strains, but it inhibited T1 (6 mg mL^{-1}) and the HDAC null mutants. The *hda1*Δ and *hda1*Δ/*hos2*Δ strains showed an inhibitory response similar to T1.

The *S. aureus* isolate UzK crude extract inhibited *C. neoformans* H99 at the concentration of 7 mg mL^{-1}, the T1 strain at 6.5 mg mL^{-1} and the HDAC null mutants at 4 mg mL^{-1}. *C. gattii* NIH198 growth was inhibited by 6 mg mL^{-1} of this extract.

The *hda1*Δ (2.5 mg mL^{-1}) and *hda1*Δ/*hos*2Δ (2 mg mL^{-1}) mutants were more sensitive to the *S. aureus* isolate UzM crude extract than the other *C. neoformans* strains (6.5 mg mL^{-1}). This extract was also effective against *C. gattii* NIH198 (4 mg mL^{-1}).

Discussion

Actinobacteria are the most important source of antimicrobial compounds, including antifungals. In this view, the screening of actinobacteria capable of producing new antimicrobial compounds represents a continuous effort (THANGAPANDIAN; PONMURUGAN; PONMURUGAN, 2007; ZHAO et al., 2012; ZOTHANPUIA et al., 2018).

Medicinal plants are known to produce a large variety of bioactive substances. Due to the long term association between microorganisms and medicinal plants through evolution, the former also take part in plant metabolic pathways and thus modulate their own metabolism. Therefore, in recent years, actinobacteria from medicinal plants have been considered a promising source of bioactive metabolites (GOLINSKA et al., 2015). However, in Asia, the studies are limited if one considers the centuries of traditional medical practise with herbal remedies, like the Ayurveda, in India (THANGAPANDIAN; PONMURUGAN; PONMURUGAN, 2007; QIN et al., 2009; ZHAO et al., 2012).

In this study, four actinobacteria isolates were purified from the rhizosphere of the medicinal plants *H. annuus* (a wild sunflower), *P. pinnata* (common name "karanj") and *Z. mauritiana* (common name "ber"). These isolates correspond to *S. gancidicus* (Ha1), *S. griseoruber* (Pp1) and to *S. aureus* (isolates UzK and UzM). Even though we still have to refine the phylogenetic studies, we believe these isolates could correspond to new varieties or strains since the species were previously described from far distant distinct environments.

Cryptococcus causes severe infection in immunocompromised hosts. There is an increasing concern about the infection of immunocompetent individuals, particularly for *C. gattii* (CHAYAKULKEEREE; PERFECT, 2006), and also on the selection of drug-resistant clinical isolates (NASCIMENTO et al., 2017).

Studies on the effect of actinobacteria-derived compounds effective against *Cryptococcus* growth are limited. VARTAK and colleagues (2014a) purified a new polyene macrolide compound (PN00053) from *Streptomyces* sp. (MTCC-5680), isolated from northern India, which exhibited antifungal activity against *C. neoformans*, *in vitro*. The ethyl acetate extract of *Streptomyces* sp. DA3-7, isolated from the Saudi Arabian desert, abolished *C. neoformans* growth at the concentration of 31.25 μg mL^{-1} (NITHYA et al., 2018).

In the present study, we evaluated the antifungal activity of crude extracts obtained from *Streptomyces* spp. isolated from the rhizosphere of the Indian traditional medicine plants against *C. neoformans* reference strain H99, fluconazole-resistant strains T1 and 89–610, *C. gattii* NIH198 and HDAC gene deletion strains (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ and *hda1*Δ+HDA1).

The crude extracts were effective in controlling *Cryptococcus* growth, with MICs ranging from 2 to 8 mg mL^{-1} (Table 1). *S. aureus* isolates UzK and UzM, which inhibited all the *Cryptococcus* strains, presented a prominent anti-cryptococcal activity. Inhibition was not observed with *S. gancidicus* Ha1 extract against *C. gattii* NIH198. No inhibition was observed either for *S. griseoruber* Pp1 extract against *C. neoformans* H99, the fluconazole-resistant 89–610 strain, and *C. gattii* NIH198.

It will be interesting to identify and characterize the molecules in the crude extract of *S. griseoruber* isolate Pp1 which did not inhibit the *C. neoformans* H99 reference strain but inhibited the growth of the fluconazole-resistant strain T1. Few studies indicated *Streptomyces* metabolites as promising agents against fluconazole-resistant *Candida* spp. (SPADARI et al., 2013; VARTAK et al., 2014b). Ouargli and colleagues 2015 demonstrated that *Streptomyces* isolates, from different sources in Nigeria, were effective against 5-fluorocytsine, azole or echinocandins resistant *Cryptococcus* strains.

Curiously, *C. gattii* NIH198 growth was inhibited only by the crude extract of *S. aureus* isolates UzK and UzM (Table 1). Previously, Amorim and colleagues demonstrated the antifungal activity of *S. ansochromogene* against *C. gattii* ATCC 24065 (AMORIM et al., 2020).

From the species we obtained, only *S. gancidicus* was previously reported as a producer of anti-cryptococcal compounds. RAUBITSCHEK and colleagues (1952) described a fungicidin type antifungal agent which inhibited the growth of *C. albicans*, *C. neoformans*, and of the yeast phase of *B. dermatitidis*. DALITZ and collegueas (2017) reported the antifungal activity against *Exophiala*, *Rhinocladiella* and *Cladosporium* of a *S. aureus* isolate obtained from marine sediments from Ilha do Mel, State of Parana, Brazil.

The crude extracts of four non phylogenetically classified *Streptomyces* isolates obtained from Indian medicinal plants, different from the ones we studied here, were able to inhibit the growth of *C. neoformans* (THANGAPANDIAN; PONMURUGAN; PONMURUGAN, 2007) and *C. albicans* (GOHAIN et al., 2019)

BACON et al. (1968) demonstrated the ability of *Streptomyces* culture filtrate to lyse the cell walls of *Cryptococcus* and *Schizosaccharomyces* by hydrolyzing α(1–3) glucan. Also, according to SOLECKA et al. (2012), the antifungal activity of actinobacteria metabolites involves the lysing of the fungal cell wall. c

Histone acetylation and deacetylation play a crucial role in chromatin dynamics and are orchestrated by the activities of opposing effect enzymes: the histone acetyltransferases (HATs) and histone deacetylases (HDACs). (EKWALL, 2005). Previously, HDAC genes have been linked to drug susceptibility and/or pathogenicity in *C. albicans* (LI et al., 2015) and *C. neoformans* (BRANDÃO et al., 2015, 2018; RANJAN et al., 2021).

Actinobacteria are also an important source of commercially available drugs which can modulate epigenetic states. The short-chain fatty acid sodium butyrate (NaBut) was initially identified as a non-specific HDAC inhibitor produced by the degradation of dietary fibres by bacteria (CANDIDO; REEVES; DAVIE, 1978).

Trichostatin A (TSA) is a HDAC inhibitor firstly isolated from *Streptomyces hygroscopicus*. Later, the TSA analogues trichostatic acid, JBIR-109, JBIR-110, JBIR-111 were obtained from *Streptomyces* RM72. JBIR-17 was isolated from *Kerria japonica*. Similar to TSA, these analogues also inhibited human HDAC. *S. platensis* is also a source for suberoylanilide hydroxamic acid, another HDAC inhibitor (Reviewed by CONTE et al., 2020). These data suggest that new epigenetic modulators can be screened from different actinobacteria.

In our study, we investigated the impact of the crude extracts of *Streptomyces* spp. obtained from Indian medicine plants on the growth of *C. neoformans* histone deacetylase gene deletion null mutants (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ and *hda1*Δ+ HDA1) (Table 4).

Overall, the HDAC gene deletion strains were more sensitive to growth inhibition by actinobacteria crude extracts than the H99 reference strain (Table 1). To the crude extract of *S. gancidicus* isolate Ha1, only the *hda1*Δ/*hos2*Δ double mutant presented increased sensitivity. The crude extract of *S. griseoruber* isolate Pp1 was particularly active against the *hda1*Δ and *hda1*Δ/*hos2*Δ strains. *S. aureus* isolate UzK similarly inhibited the different null mutant strains. These strains were more susceptible than H99. The crude extract of *S. aureus* isolate UzM presented a pronounced inhibitory effect on the growth of the *hda1*Δ and *hda1*Δ/*hos2*Δ strains.

Previously, in our laboratory, we demonstrated an increased susceptibility of the aforementioned mutant strains to trichostatin A and to hydralazine, a DNMTi (RANJAN et al., 2021). We speculate that the increased susceptibility of the *C. neoformans* HDAC mutants is

related to the alteration in chromatin remodelling machinery. It is also possible that the crude extracts present additional HDAC or DNMT inhibitors which could further affect cell growth.

Conclusion

Natural products of microbial origin are widely considered as alternative sources of metabolites which could be employed in the therapy of fungal infections, especially those involving drug-resistant isolates. In the present study, we isolated four *Streptomyces* spp. (*S. gancidicus*, *S. griseoruber* and *S. aureus*) from the rhizosphere of *H. annuus*, *P. pinnata* and *Z. mauritiana*, commonly employed in Indian folk medicine. The *Streptomyces* spp. crude extracts presented antifungal activity against *Cryptococcus* spp., especially fluconazole-resistant and histone deacetylase gene deletion strains. Our data point out to the potential of the *Streptomyces* spp. as sources of novel metabolites for the designing of alternative antifungal therapeutics against *Cryptococcus*.

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Supplementary Tables

Table S1: Cultural characteristics of actinobacterial isolates grown at 28 °C on AGA plates

Isolates	Pigments		
	Substrate mycelia	Aerial mycelia	Soluble pigment
Ha1	White	Grey	absent
Pp1	White	Light brown	Light brown
UzK	Whitish brown	Dark brown	Dark brown
UzM	Whitish brown	Dark brown	Dark brown

Table S2. pH values of the crude extracts and dry cell biomass after fermentation

Isolate	pH	Dry biomass (mg)
Control	7.29	-
Ha1	8.11	838
Pp1	8.48	166
UzK	8.39	326
UzM	8.76	489

Table S3: Minimum inhibitory concentrations (MIC) values of crude extracts obtained from actinobacteria *S. gancidicus* isolate Ha1 (SgdHa1), *S. griseoruber* isolate Pp1 (SgrPp1), *S. aureus* isolates UzK (SaUzK) and UzM (SaUzM) isolates against the *C. neoformans* H99, T1 and 89–610 and *C. gattii* NIH198 strains, and for the *C. neoformans* histone deacetylase null mutants. 2X RPMI media was used.

Strains	Minimum inhibitory concentrations (mg mL^{-1})			
	SgdHa1	SgrPp1	SaUzK	SaUzM
C. neoformans H99	>40	8	**7**	8
C. neoformans T1	>40	8	7	8
C. neoformans 89–610	>40	8	7	8
*hda1*Δ	>40	8	8	3.5
*hos2*Δ	>40	8	8	6
*hda1*Δ/*hos2*Δ	>40	8	8	3.5
*hda1*Δ+HDA1	>40	>40	8	8
C. gattii NIH198	>40	>40	7	8

Supplementary Figures

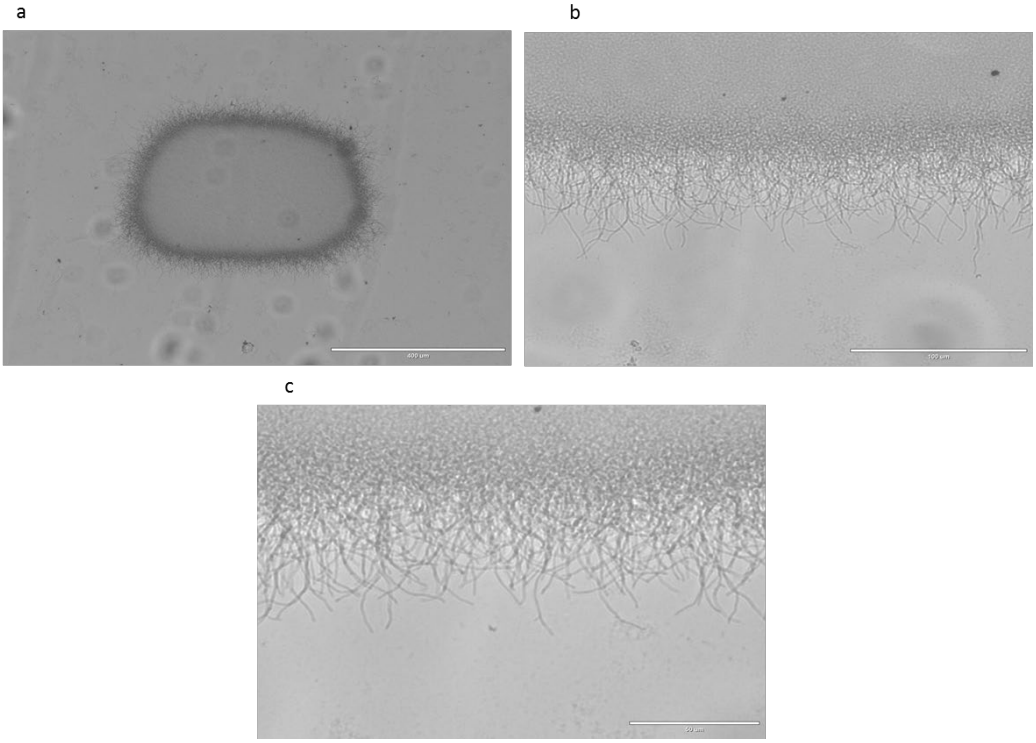

Figure S1: Optical microscopy of *S. aureus* UzK. (a) under a 20x objective (b) under 40x objective. (c) under 60x objective

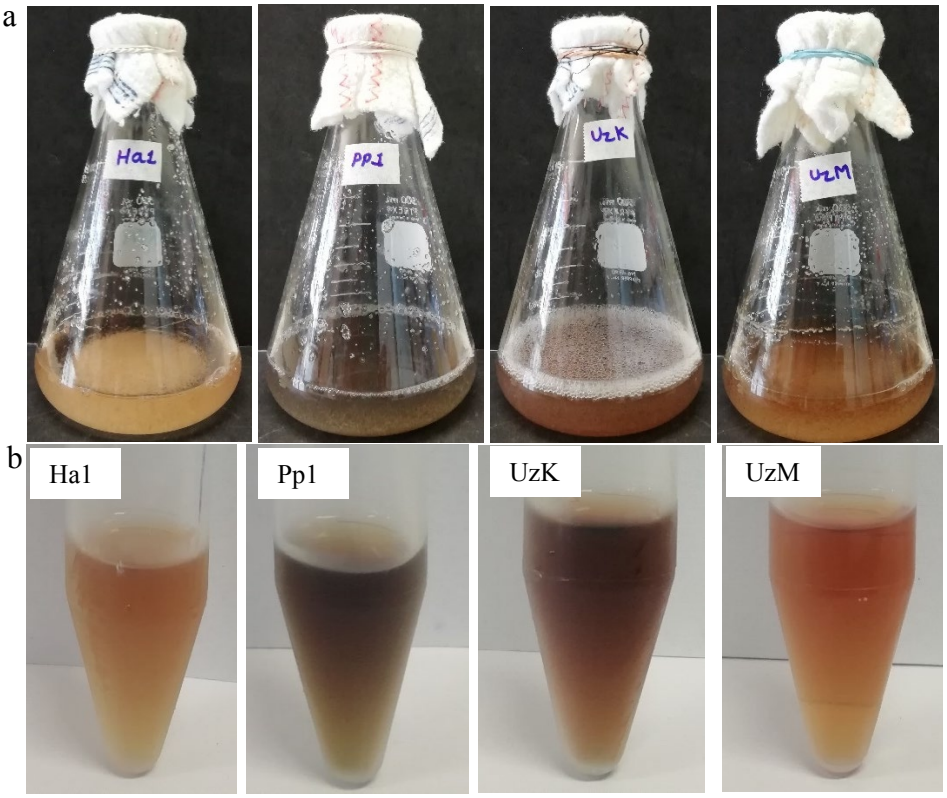

Figure S2. a) Photographs of cultures grown at 28 °C, 150 rpm, for 14 days in fermentation broth. b) Resuspended crude extract powder at the concentration of 80 mg mL^{-1}.

CHAPTER III

The growth of newly isolated fungi from the Brazilian Amazon region is inhibited by actinobacteria from the rhizosphere of Indian Traditional Medicine plants

3. Introduction

3.1 Fungi associated with iron ore formations

Destruction of natural habitats is an ever-increasing alarming threat in Brazil. Conservation units are effective barriers against them (DA SILVA; RYLANDS; DA FONSECA, 2005). The Carajás National Forest, which covers more than one million hectares, is part of a mosaic of conservation units located in the Amazonian region, in Pará State, Brazil.

Deforestation for mining activities in Amazon intensified in the last 50 years. The Carajás National Forest holds the world's largest iron ore deposits. Iron ore from this region covers 3% of the landscape and is surrounded by Savanna vegetation (ARDENTE et al., 2016).

In general, underground iron ore mines present an environment similar to the one of caves: total darkness, high humidity, low temperature and elevated concentration of metals. These extreme environments are different from the external territories and harbours unique microorganisms.

Filamentous fungi are a diverse group of microorganisms which are important for nutrients cycling in the soil, decaying organic matter, enzymes and other industrial inputs, antibiotics and other drugs. Some can cause serious plant, animals and human diseases, especially in immunocompromised individuals (Reviewed by HYDE et al., 2018).

In caves and mining areas, fungi that pose the greatest risk to human health correspond to members of the genus *Histoplasma*, *Blastomyces*, *Coccidioides* and *Paracoccidioides*. The opportunists *Pneumocystis*, *Candida*, *Rhizopus*, *Mucor*, *Fusarium* and *Aspergillus* are also present (KÖHLER et al., 2017 DENHAM; WAMBAUGH; BROWN, 2019). Comorbidities associated with the so-called "black yeasts" (genera *Fonsecaea*, *Rhinocladiella*, *Phialophora* and *Exophiala*) draw the attention of the scientific community (CHOWDHARY; PERFECT;

DE HOOG, 2015). Several of these fungi, notably *H. capsulatum*, are commonly found in cave environments, usually in association with bat excreta and other animals.

As the conditions of light, humidity, air and water flow, and temperature are usually different from those found in nearby open environments, it is reasonable to consider that the mycobiota of the caves or subterranean iron ore mines present unique organisms. ZHANG and colleagues (2017) estimated that about 1150 species of fungi, belonging to 550 genera, have been described in cave and mine environments around the globe.

There are many studies on the population diversity of microorganisms in caves and/or mines of North America (MICALIZZI et al., 2017; HELD; SALOMON; BLANCHETTE, 2020; VISAGIE et al., 2020). Thus, it is plausible to affirm that subterranean iron ore mines of The Carajás National Forest may be harbouring important and unique microbiological biodiversity.

3.1.1 *Fusarium*

Fusarium spp. are commonly found in the soil, plants, cereals and grains. The genus comprises more than 50 species. Generally, infections due to *Fusarium* species are referred as fusariosis. *F. solani* complex, *F. verticilliodes*, *F. oxysporum* and *F. proliferatum* are the main causative agents. *F. solani* is the most frequent (NUCCI; ANAISSIE, 2007; Reviewed by EGBUTA; MWANZA; BABALOLA, 2017).

Fusarium species are intrinsically resistant to most of the azoles and/or echinocandins. Multidrug resistant *Fusarium* species poses threat to human health (Fisher et al., 2020). In immunocompromised individuals, the mortality rates due to fusariosis are estimated in 50 to 70% mortality. Generally, the *F. solani* species complex is found in all clinical infections (NUCCI; ANAISSIE, 2007; MUHAMMED et al., 2013;HOF, 2020).

3.1.2 *Mucorales*

Mucorales are the second most common fungi after *Aspergilli*. They can cause mucormycosis, which is associated with high mortality and morbidity. *Mucorales* comprise 11 genera and more than 27 species associated with human infections. *Rhizopus*, *Mucor*, *Rhizomucor* and *Lictheimia* are the most common mucormycosis agents (PRAKASH; CHAKRABARTI, 2021; RODEN et al., 2005). Diabetes mellitus is the main risk factor for mucormycosis in Asian countries, while organs transplantation and malignancies represent the main susceptibility comorbidities in Europe and USA patients (CHAKRABARTI et al., 2006; SKIADA et al., 2011;CHEGINI et al., 2020).

Mucorales are highly drug resistant and this problem is increasing worldwide. Amphotericin B, isavuconazole and posaconazole are the only drugs approved for the mucormycosis treatment (MARTY et al., 2016). The Leading International Fungal Education (LIFE) portal estimated around 910, 000 mucormycosis cases globally (BONGOMIN et al., 2017). CAETANO and collaborators (2019) studied the prevalence of *Mucorales* in different indoor environments in Portugal. Sixteen isolates of *Mucor* sp., *Rhizopus* sp. and *Rhizomucor* sp. were resistant to voriconazole (1 mg ml^{-1}), while four isolates of *Mucor* sp. and *Rhizopus* sp. were refractory to itraconazole (4 mg mL^{-1}).

3.1.3 *Penicillium*:

Though *Penicillium* species are highly diverse and widely disseminated in environment, only few of them can be pathogenic to humans. Infections due to *Penicillium* are mainly referred as penicilliosis or talaromycosis. *P. citrinum*, *P. chrysogenum*, *P. digitatum*, *P. expansum* and *P. marneffei* are associated with penicilliosis (WALSH et al., 2004;LASS-FLÖRL; CUENCA-ESTRELLA, 2017). After tuberculosis and cryptococcosis, penicilliosis

emerged as the third most common opportunistic disease in HIV/AIDS patients (CAO et al., 2011; LE; THANH; THWAITES, 2020).

Therapeutic options to penicilliosis treatment are limited. ALCAZAR-FUOLI and colleagues (2009) described azole resistant *P. citrinum* and *P. minioluteum* isolates. Amphotericin B and echinocandins were effective in controlling the growth of *Penicillium*. There is no guideline for testing the *P. marneffei in vitro* drugs susceptibility profile, and for the duration of treatment and prophylaxis among HIV-uninfected patients (Reviewed by CAO; XI; CHATURVEDI, 2019).

3.1.4 *Chaetomium*

Chaetomium spp. are ascomycetes found in the environment. They are able to completely degrade cellulose and to produce bioactive metabolites, such as mycotoxin, chaetoglobosin A, polysaccharide and furan derivative anti-inflammatory metabolites (FOGLE et al., 2007; KIM et al., 2020; WANG et al., 2019, 2020). More than 300 species of *Chaetomium* were described. Few are known to cause disease in human, such as *C. globosum*, *C. atrobrunneum*, *C. strumarium* and *C. perlucidum* (ATTIA; ABDEL-AZEEM, 2020).

Studies addressing *Chaetomium* spp. susceptibility profile to antifungal drugs are limited. SUN and colleagues (2019) demonstrated the antifungal activities of amphotericin B, itraconazole, voriconazole, posaconazole, caspofungin and terbinafine, either alone or in combinations against 22 *Chaetomium* isolates from the Research Centre for Medical Mycology of the Peking University, China. *Chaetomium* spp. were more sensitive to amphotericin B and to triazole than to caspofungin and terbinafine. Among triazoles, voriconazole was less effective in controlling the *Chaetomium* growth in comparison to itraconazole and posaconazole. Caspofungin was highly synergistic with itraconazole.

3.1.5 *Aspergillus*

The *Aspergillus* genus correspond to the most abundant organisms on earth. It comprises more than 300 species. Over 20 species were identified as potential human pathogens (Reviewed by LANDÍNEZ-TORRES et al., 2020). They can cause invasive aspergillosis mainly in immunocompromised individuals. Globally, more than 200 000 cases of aspergillosis are reported every year. *A. fumigatus* is the main causative agent, but species such as *A. flavus*, *A. niger* and *A. terreus* are also associated to aspergillosis (PÉREZ-CANTERO et al., 2020).

Voriconazole, liposomal amphotericin B and isavuconazole are the drugs of choice to treat aspergillosis. The extensive employment of azoles in clinical treatment and in agriculture led to increased selection of azole-resistant *Aspergillus* isolates (FARIA-RAMOS et al., 2014; SNELDERS et al., 2009; VERMEULEN et al., 2015) and this is associated with treatment failures (FUHREN et al., 2015; VAN DER LINDEN et al., 2013).

A 1.7% to 6% increase in infections provoked by itraconazole resistant *Aspergilli* was reported from 1994 to 2007 in the Netherlands (SNELDERS et al., 2008). In the United Kingdom, an increase in azole resistant *A. fumigatus* infections was reported in 2004 (5%) and in 2009 (20%) (BUEID et al., 2010). CHAKRABARTI and collaborators (2019) surveyed invasive mold infections in ICUs of 11 tertiary care centres across India. The authors reported *A. flavus* (47%) and *A. fumigatus* (39.4%) as the most common fungi isolated from patients.

Drug resistant microbes are a major concern in human health and represent a serious problem in the treatment of fungal infections. Drug resistance is driving the need for new antifungal compounds targeting unconventional drug sites. Natural products from microorganisms, including actinobacteria, correspond to a rich source of novel antifungal compounds (Reviewed by JAKUBCZYK; DUSSART, 2020).

3.2 JUSTIFICATION

Caves and mines are special ecological niches that harbour rare microflora, including fungi. Microscopic fungi are understudied in iron ore mining especially in South America. Data on filamentous fungi from caves and/or iron ore mining are very limited and could contribute to the studies on the biology of microorganisms from extreme environments. This study aims to isolate filamentous fungi from subterranean iron ore mining areas of The Carajás National Forest, Brazil and to assess their susceptibility to the antifungal properties of *Streptomyces gancidicus*, *S. griseoruber* and *S. aureus*

3.3 Objectives

a) To isolate and to identify filamentous fungi from subterranean iron ore mines of The Carajás National Forest (Pará, Brazil).

b) To assess their susceptibility to the antifungal properties of *Streptomyces gancidicus*, *S. griseoruber* and *S. aureus* previously isolated from rhizosphere of Indian Traditional medicine plants (*Helianthus annuus*, *Pongami pinnata* and *Ziziphus mauritiana*).

c) To assess the susceptibility of *Chaetomium madrasense*, *Aspergillus* terreus, *A. flavus* and *A. fumigatus* to the antifungal activity of aforementioned *Streptomyces* spp.

The growth of newly isolated fungi from the Brazilian Amazon region is inhibited by actinobacteria from the rhizosphere of Indian Traditional Medicine plants

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Abstract

Caves and mines are distinct ecosystem due to the low light incidence and temperature, high humidity, elevated metals concentration and low trophic resources. It contains rare microbiomes and are greatly understudied in comparison to other environments. In this work, filamentous fungi were isolated from subterranean iron ore mines of The Carajás National Forest (Pará, Brazil) and identified from ITS and genus specific sequences as *Fusarium solani*, *F. striatum*, *Mucor irregularis*, *M. fragilis* and *Penicillium citrinum*. These isolates, described for the first time from the aforementioned region, *Chaetomium madrasense* and *Aspergillus* spp. were co-cultured with *Streptomyces gancidicus*, *S. griseoruber* and *S. aureus*, previously obtained from the rhizosphere of Indian Traditional Medicine Plants.

The *Streptomyces* species inhibited the radial growth of *Fusarium* spp. from 26.96 to 72.52%, *M. fragilis* from 18.18 to 75.25% and *P. citrinum* from 51.39 to 100%. *C. madrasense* inhibition ranged from 13.58 to 34.16% and *Aspergillus* spp from 45.83 to 100%. These results indicate that actinobacteria from the rhizosphere of Indian medicinal plants represent a

promising source of new antifungal compounds to control infections provoked by potential opportunistic filamentous fungi.

Keywords: Filamentous fungi, Amazonian iron ore mining area fungi, Actinobacteria, Indian Traditional Medicine plants, antifungal activity.

Introduction

Subterranean iron ore mining and caves are rare ecosystems presenting distinctive microbiomes due to low light incidence and temperature, high humidity, elevated metals concentration and low trophic resources (CULVER, 1982; FERREIRA, 2005; HELD; SALOMON; BLANCHETTE, 2020). Numerous opportunistic pathogens such as *Pneumocystis*, *Candida*, *Rhizopus*, *Mucor*, *Fusarium* and *Aspergillus* were isolated from caves and/or mines worldwide (KÖHLER et al., 2017; MICALIZZI et al., 2017; DENHAM; WAMBAUGH; BROWN, 2019; HELD; SALOMON; BLANCHETTE, 2020; VISAGIE et al., 2020). Nonetheless, there is no report on study of fungus from iron ore mining area in the Carajás National Forest (State of Para, Brazil).

The ability of fungi to tolerate multiple stresses (polyextremotolerance) and to grow at mammal body temperature is linked to the capacity to infect human hosts. Opportunist pathogenesis and polyextremotolerance share common evolutionary history. It was postulated that the fungal traits important for pathogenicity were shaped by selection pressure under extreme conditions, outside the host organism (CASADEVALL; PIROFSKI, 2007; GOSTINČAR et al., 2018). In this view, fungi from extreme environments, such as the subterranean iron ore mining areas, could represent model organisms for understanding the phenotype plasticity which is a commonly associated to opportunistic pathogens.

Actinobacteria have attracted attention for many years due to the capability of producing compounds that can inhibit filamentous fungi growth (CACERES et al., 2018; YANG et al., 2019; PELLAN et al., 2020; PENG et al., 2020; PRIMAHANA et al., 2020; SIDDHARTH; VITTAL; WINK, 2020). The investigation on new antifungal substances derived from actinobacteria is particularly relevant to tackle the increasing problem of multiple drug resistance by clinical isolates.

KHEBIZI and colleagues (2017) studied the inhibitory potential of *Streptomyces gancidicus* isolated from Saharan soil sample, Algeria, against *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. Antifungal compounds were identified and named oligomycin E and A.

S. alboflavus TD-1, isolated from soil, in China, produced volatile organic compounds which inhibited the mycelial growth, sporulation, and conidial germination of *A. flavus*. It also inhibited the aflatoxin B1 production by downregulating the genes involved in the biosynthesis (YANG et al., 2019). Fungichromin is a methylpentaene macrolide compound produced by *Streptomyces* sp. strain, WP-1, obtained from bark of *Pinus dabeshanensis*, also China, and it inhibited the mycelial growth and conidia germination the *Fusarium oxysporum* plant pathogen (PENG et al., 2020).

Recently, we demonstrated that crude extracts from *Streptomyces gancidicus*, *S. griseoruber* and *S. aureus* isolates obtained from the rhizosphere of *Helianthus annuus*, *Pongamia pinnata* and *Ziziphus mauritiana*, in the rural area of Rohtak (State of Haryana, India), inhibited the growth of different *Cryptococcus neoformans* strains, *C. gattii*, *Candida albicans*, *C. parapsilosis* and *C. tropicalis* yeasts (Ranjan et al., manuscript in preparation).

In face of the above, our goal in the present study was to isolate filamentous fungi from the unexplored iron ore mining area in the Carajás National Forest (State of Para, Brazil) and to evaluate their growth upon co-culture with *S. gancidicus*, *S. griseoruber* and *S. aureus*. We

have also studied the *Streptomyces* antifungal activity against *Chaetomium madrasense*, *Aspergillus terreus*, *A. flavus* and *A. fumigatus*.

Our results demonstrated that *S. gancidicus*, *S. griseoruber* and *S. aureus* inhibited the radial growth of *Fusarium striatum*, *F. solani*, *Mucor irregularis M. fragilis* and *Penicillium citrinum*, isolated from the Carajás National Forest, *C. madrasense* and *Aspergillus* spp.

Materials and methods

Fungi collection from the Carajás National Forest (Daniel Maiolino, personal communication)

Samples were collected from the iron ore plateau of the Carajás National Forest (State of Pará, Brazil; -6,042143 S/-50,208115 W) (Supplementary Figure 1a). Permission for the study within this region was taken under the project "Underground fauna ecology and population genetic structure as an indicator of habitat connectivity of troglobic species in the Carajás iron system" (Ecologia da fauna subterrânea e estrutura genética populacional como indicador de conectividade de habitat de espécies troglóbias no sistema ferrífero de Carajás; Project number: SISBIO 62000). The N3 iron ore plateau of the forest was selected for the study (Supplementary Figure 1b).

To study the underground fauna of the ferruginous region of the site, a trap was developed according to HALSE and PEARSON (2014). These traps were made of PVC tubes (300 x 75 mm, with access holes on the sides) (Supplementary Figure 1c). Five traps were placed in each borehole, with the spacing of 5 meters between them, containing one of the two types of baits: 1) standard bait (wet litter collected previously on the spot, sterilized in an oven at 90 ºC for 1 hour), and 2) enriched bait (sterilized litter mixed supplemented with bovine liver). These traps were introduced in the drill holes 25 meters deep (Supplementary Figure

1d). The traps were put into the soil in August 2019 for approximately 8 weeks. After this period, the traps were removed from the soil, individually packed and analysed in the entomology laboratory of the Federal Rural University of Amazon – UFRA.

The entrances of a few traps were blocked by fungus-like structures, as shown in Supplementary Figure 1e. These structures were inoculated onto solid YPD (2% yeast extract; 1% peptone; 2% glucose 2% agar) containing 200 μg mL^{-1} ampicillin, incubated at 28 °C for a few days and then taken to the Laboratory of Gene Regulation and Mutagenesis, Department of Genetics and Morphology/IB, University of Brasilia. The material was spread to single colonies on solid YPD, incubated at 28 °C and the plates were observed daily for fungal growth. Morphologically distinct colonies were subcultured to purity. The purity of the fungal isolates was confirmed under the light microscope. Pure cultures were stored at -80 °C.

DNA extraction, PCR amplification and phylogenetic analyses

Pure fungal isolates were inoculated in 3 mL of liquid YPD at 28 °C, 150 rpm, for 3 to 5 days. Extraction of DNA, PCR amplification details and taxonomical analyses were performed as described (Ranjan et al., manuscript in preparation). Primers used in this study are listed in Supplementary Table S1.

Thermotolerance assay

Pure isolates samples were spread on the entire surface of YPD plates and incubated at 28 °C for 5 to 7 days, to create a lawn of mycelium. After incubation, seven mm diameter mycelial agar plugs were placed upside down at the centre of a fresh YPD plate. Plates were incubated at 28 °C, 37 °C or 42 °C. For assessment of the radial growth, the radius of each colony was measured daily until it covered the plate. Five replicates of each fungus, for each temperature, were performed.

***Streptomyces* isolates**

S. gancidicus, *S. griseoruber* and *S. aureus* isolation from the rhizosphere of the Indian traditional Medicine plants (*H. annuus*, *P. pinnata* and *Z. mauritiana*), their morphological characterization and phylogenetic analyses was described elsewhere (Ranjan et al., manuscript in preparation).

Confrontation assay

Confrontation assays were performed to observe the antagonistic potential of the previously obtained actinobacteria isolates against different filamentous fungi: 1) four species obtained from the Carajás National Forest iron ore plateau, 2) *Chaetomium madrasense* isolated as a laboratory contaminant at the Department of Microbiology, Maharshi Dayanand University (Rohtak, India) and 3) three *Aspergillus* spp. (*A. flavus*, *A. fumigatus* and *A. terreus*) generously provided by Prof. Edivaldo Ximenes Ferreira Filho (Laboratory of Enzymology, University of Brasilia, Brazil).

Seven mm of agar plugs of actinobacteria cultures were inoculated on the surface of Mueller-Hinton agar (MHA) plates (0.2% beef extract; 1.75% acid hydrolysate of casein; 0.15% starch 1.7% agar, pH-7.3) and incubated at 28° C, for 14 days, for growth and metabolites secretion. After that, seven mm diameter plugs of fungal strains cultures were inoculated at a distance of 1.0-1.5 cm from the margin of the actinobacteria colony; plates were further incubated for 5 days. As control, fungal colony plugs were placed in MHA plates without actinobacteria. The radial growth of the fungi mycelia was measured. The percentage of inhibition of radial growth (PICR) was determined according to SÁNCHEZ-GARCÍA et al. (2019), PICR= (R1-R2)/R1*100, where R1 is the radius of fungal growth in the control plate

and R2 is the radius of fungal growth in confrontation plate. Experiments were performed as technical triplicates.

Statistical analysis

Graph and statistical analysis for thermotolerance assay was performed with the GraphPad Prism 6.0 software (California, USA), with 0.05 level of significance determined by two-way ANOVA and Tukey's multiple comparison test. The error bar represents the standard deviation.

Results

Macroscopic and microscopic features of the fungi isolated from the Carajás National Forest iron ore plateau

Eight fungal isolates (Rosa, Branco2, Branco.branco, Branco.verde, Cinza.cinza, Cinza.branco, Amarelo and Verde) were obtained from the subterranean iron ore plateau of the Carajás National Forest. The isolates were streaked to pure cultures and examined based on macroscopic and microscopic characteristics (Table 1, Figure 1, Supplementary Figure 2).

During the actinobacteria purification in India, a dark colour filamentous fungus, which grew and sporulated easily, was found as a plate contaminant. Since it was inhibited by the actinobacteria growth, it was included in our antimicrobial activity tests and was named Kala (Table 1, Figure 1, Supplementary Figure 2).

Table 1: Cultural and morphological characteristics of fungal isolates grown at 28 °C on YPD plates

Isolates	Cultures general characteristics	Morphological characteristics
Rosa	Slow growth; white cottony aspect from the front view, yellow colour from the rear.	Septate hypha with branching, sickle shaped macroconidia.
Branco2	Slow growth, white cottony aspect from the front view, yellow colour from the rear.	Septate hypha with branching, sickle shaped macroconidia.
Branco.branco	Slow growth, white cottony aspect from the front view, yellow colour with black spots from the rear.	Septate hypha with branching, distinctive sickle shaped macroconidia.
Branco.verde	Slow growth; white cottony aspect from the front view, yellow colour with black spots from the rear.	Septate hypha with branching, sickle shaped macroconidia.
Cinza.cinza	Rapid growth; white colour from the front view, which turns into grey upon sporulation; orange/yellow colour from the rear.	Non-septate, branched hyphae, globose to sub-globose sporangia.
Cinza.branco	Rapid growth; white colour from the front view, which turns into grey upon sporulation; orange/yellow colour from the rear view.	Non-septate, branched hyphae, globose to sub-globose sporangia.
Amarelo	Rapid growth, white cottony aspect from the front view, which turns into yellow upon sporulation; white to pale colour from the rear	Non-septate, branched hyphae, globose to sub-globose sporangia.
Verde	Slow growth; yellow/orange colour from the front view, dark brown from the rear. It produces liquid droplets on the colony surface.	Septate hypha with branching, globose to sub-globose conidia.

Kala	Rapid growth; white aerial hyphae which turn into black upon sporulation; black colour from the rear view. Abundant spores formation.	Septate hypha with branching.

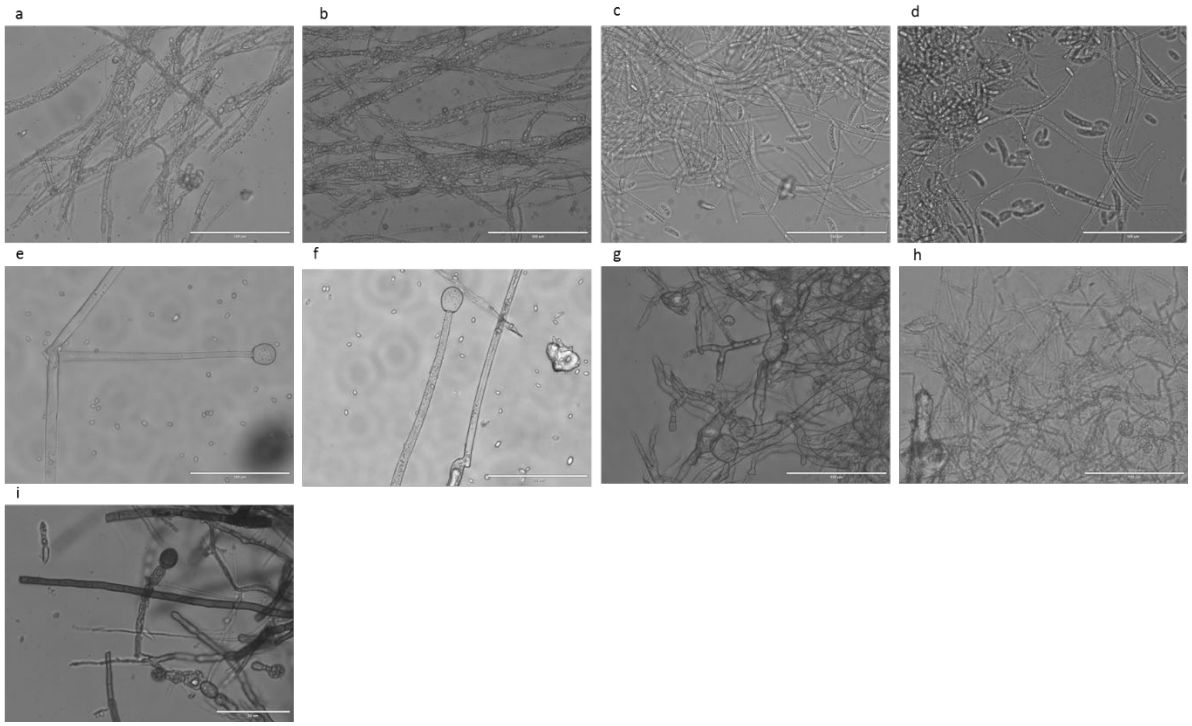


Figure 1: Photomicrographs (40X magnification) of pure cultures. a) Rosa, b) Branco2, c) Branco.branco, d) Branco.verde e) Cinza.cinza, f) Cinza.branco, g) Amarelo, h) Verde and i) Kala

Phylogenetic analysis of the filamentous fungi isolated from the Carajás National Forest iron ore plateau and of isolate Kala

From the fungal ITS sequences analysis, Rosa and Branco2 were identified as *Fusarium striatum*, which belongs to FSSC (*Fusarium solani* species complex) 21, Branco.branco and Branco.verde were identified as *Fusarium solani* and belong to FSSC 24a (Figure 2). The analysis of these isolates using *Fusarium* specific translation elongation factor (*tef1*) gene sequences further confirmed the classification (Supplementary Figure S3).

Fungal and *Mucor* specific ITS region analyses identified Cinza.cinza and Cinza.branco as *Mucor fragilis*, and Amarelo as *Mucor irregularis* (Figure 3 and Supplementary Figure S4).

Fungal ITS sequence analysis identified Verde as *Penicillium citrinum* (Figure 4), confirmed by beta-tubulin gene (*benA*) sequence analysis (Supplementary Figure S5).

Kala was identified as *Chaetomium madrasense* based on the fungal ITS sequence (Figure 5).

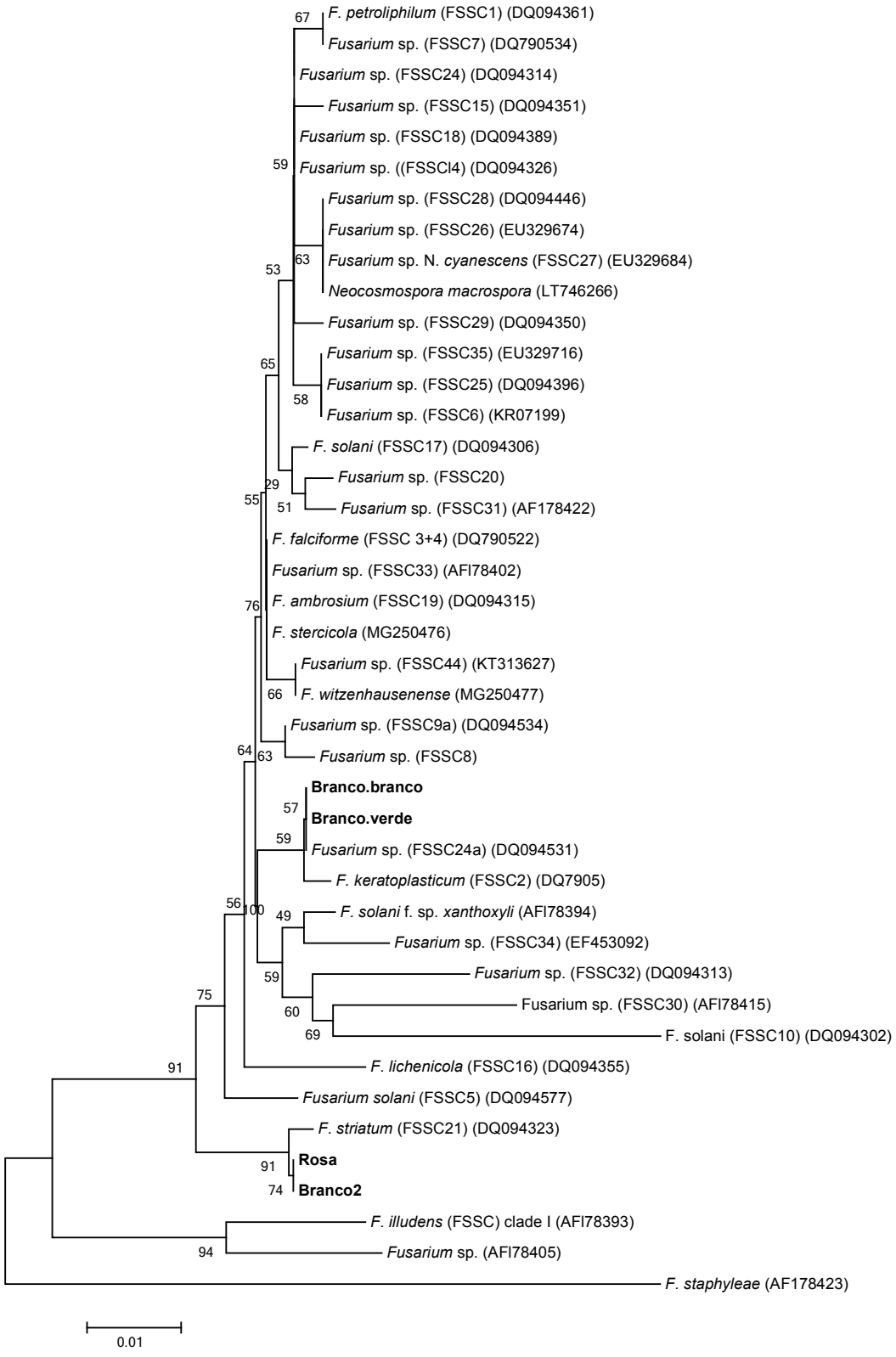


Figure 2: Phylogram showing the position of the Branco.branco, Branco.verde, Rosa and Branco2 isolates in relation to other *Fusarium solani* species complex (FSSC) based on ITS sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Fungi isolates from this study are in bold. Accession numbers are presented in parenthesis.

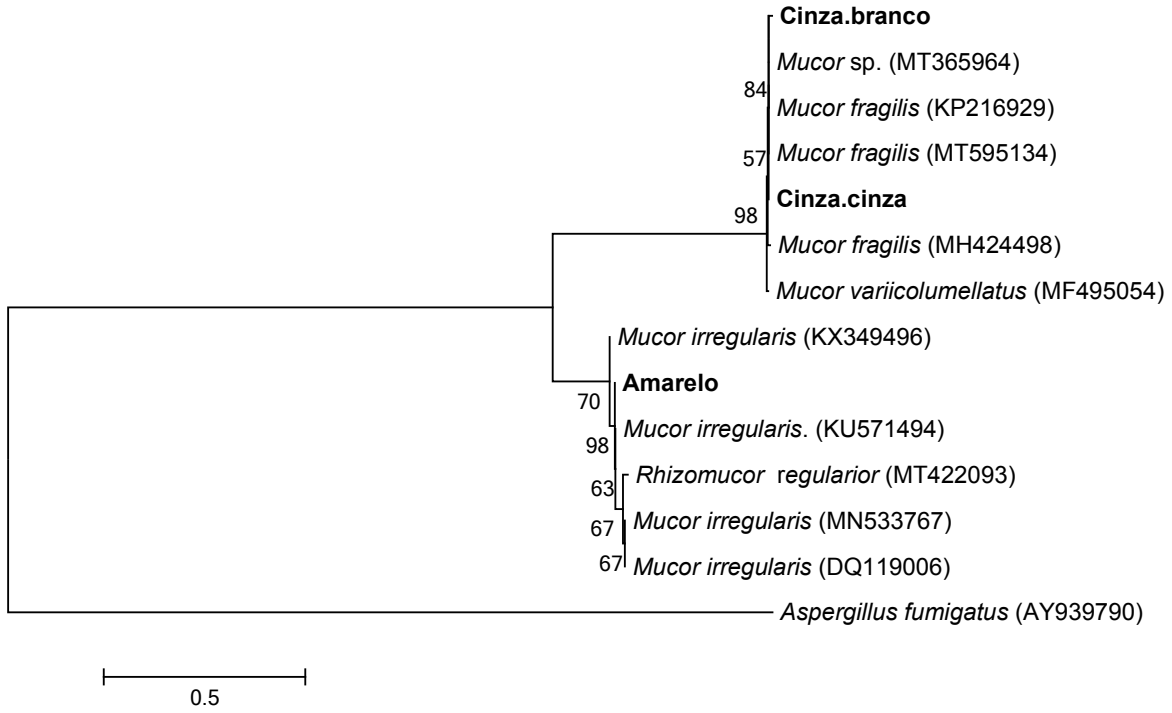


Figure 3: Phylogram showing the position of the Cinza.branco, Cinza.cinza and Amarelo isolates in relation to other *Mucor* spp. based on ITS sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Fungi isolates from this study are in bold. Accession numbers are presented in parenthesis.

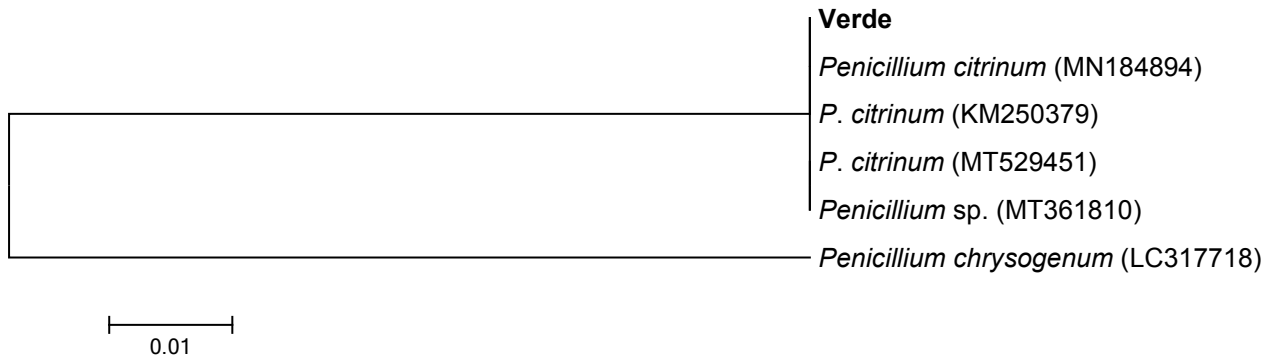


Figure 4: Phylogram showing the position of the Verde isolate in relation to other *Penicillium* spp. based on ITS sequences. The tree is constructed based on maximum likelihood analysis. Verde appears in bold. Accession numbers are presented in parenthesis.

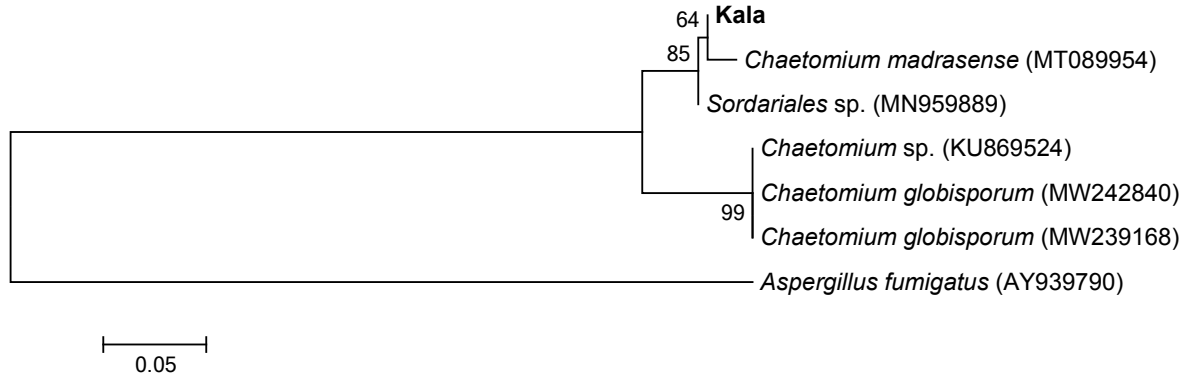


Figure 5: Phylogram showing the position of the Kala isolate in relation to other *Chaetomium* spp. based on ITS sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Kala appears in bold. Accession numbers are represented in parenthesis.

One isolate of *F. striatum* (Rosa), one of *F. solani* (Branco.branco), one of *Mucor fragilis* (Cinza.cinza), *P. citrinum* (Verde) and *C. madrasense* (Kala) were selected for further studies.

Accession numbers of the fungi nucleotide sequences

The ITS and functional ORF fungal sequences obtained in the present study were submitted to the NCBI under the accession numbers: a) *Fusarium striatum* isolates, Rosa (MW680645 for ITS and MW874647 for *tef1*) and Branco2 (MW680646 for ITS and MW887656 for *tef1*), b) *F. solani* isolates, Branco.branco (MW680647 for ITS and MW929087 for *tef1*) and Branco2 (MW680646 for ITS and MW887656 for *tef1*), c) *Mucor fragilis* isolates, Cinza.cinza (MW680650 for ITS and MW683306 for *Mucor* specific ITS) and Cinza.branco (MW680651 for ITS and MW683307 for *Mucor* specific ITS), d) *M. irregularis* (MW680649 for ITS and MW683322 for *Mucor* specific ITS), e) *Penicillium citrinum* isolate Verde (MW680652 for ITS and MW864543 for *benA*), and f) *Chaetomium madrasense* isolate Kala (MW916097 for ITS region).

Thermotolerance assays

The filamentous fungi from Amazonia subterranean ferruginous areas and the *C. madrasense* isolate Kala were subjected to growth at 28 °C, 37 °C and 42 °C (Figure 6).

At 28 °C, the mycelial growth rate of *F. striatum* isolate Rosa (4.92 mm day^{-1}) and *F. solani* isolate Branco.branco, both belonging to FSSC, was similar (4.98 mm day^{-1}). *M. fragilis* isolate Cinza.cinza grew radially at the rate of 5.96 mm day^{-1}. *P. citrinum* isolate Verde showed the lower growth rate (1.96 mm day^{-1}), while *C. madrasense* isolate Kala demonstrated the highest radial growth rate at this temperature (13.73 mm day^{-1}).

At 37 °C, the growth rate of the *F. striatum* Branco.branco isolate (1.28 mm day^{-1}) was higher than for the *F. solani* Rosa isolate (0.61 mm day^{-1}). Among all the species from the iron ore plateau, *M. fragilis* isolate Cinza.cinza presented the highest radial growth (1.73 mm day^{-1}). Curiously, radial growth of *P. citrinum* isolate Verde stopped after 17 to 22 days of incubation at 37 °C (radius 13 to 16 mm). *C. madrasense* isolate Kala demonstrated the highest radial growth at this temperature (14.40 mm day^{-1}) and was the only one to grow at 42 °C (7.47 mm day^{-1}).

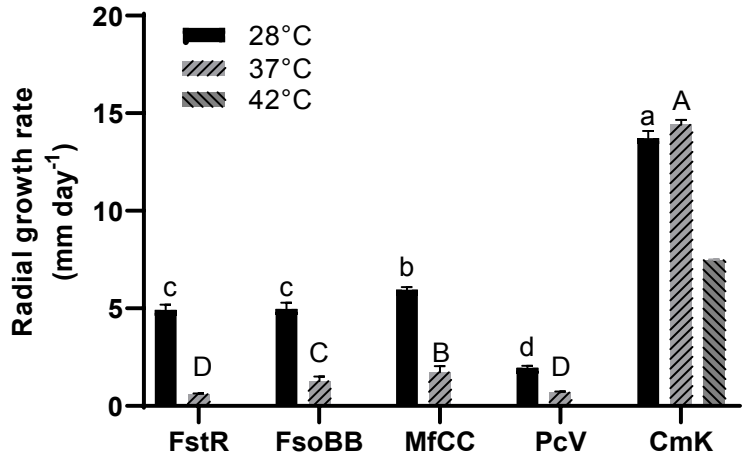
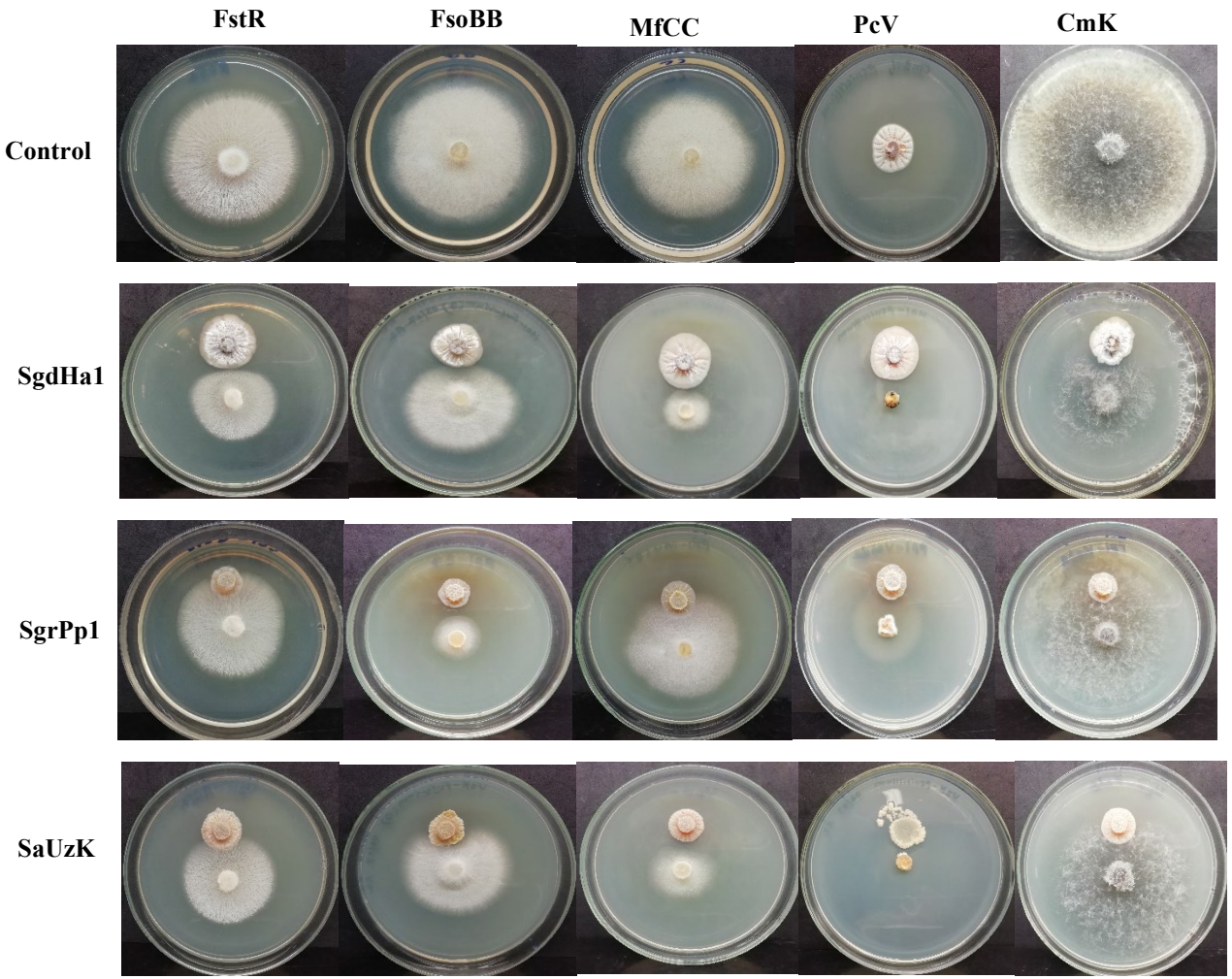


Figure 6: Fungi radial growth rates at different temperatures. Equal letters represent no statistical differences between temperatures ($p < 0.05$). FstR: *F. striatum* isolate Rosa, FsoBB: *F. solani* isolate Branco,branco, MfCCCC: *M. fragilis* isolate Cinza.cinza, PcV: *P. citrinum* isolate Verde and CmK: *C. madrasense* isolate Kala.

Confrontation assays

The filamentous fungi isolated from the subterranean iron ore in the Carajás National Forest, the *C. madrasense* isolate Kala and *A. terreus*, *A. fumigatus* and *A. flavus* were co-cultured with the *Streptomyces* isolates on Mueller-Hinton agar (MHA) medium.

All the *Streptomyces* species inhibited the filamentous fungi mycelial growth (Figures 7 and 8). This inhibition did not require physical contact between the species, thus suggesting metabolite production and diffusion through the solid medium. Interestingly, *C. madrasense* aerial growth increased when co-cultured with the actinobacteria.



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SaUzK

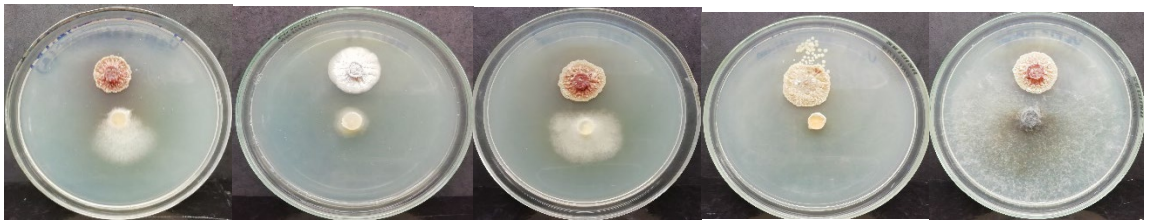

Figure 7: Anti-fungal activity of *S. gancidicus* isolate Ha1 (SgdHa1), *S. griseoruber* isolate Pp1 (SgrPp1), *S. aureus* isolates UzK (SaUzK) and UzM (SaUzM) against *F. striatum* isolate Rosa (FstR), *F. solani* isolate Branco.branco (FsoBB), *M. fragilis* isolate Cinza.cinza (MfCC), *P. citrinum* isolate Verde (PcV) and *C. madrasense* isolate (CmK) on 90 mm MHA plates. Control: fungal growth without actinobacteria.

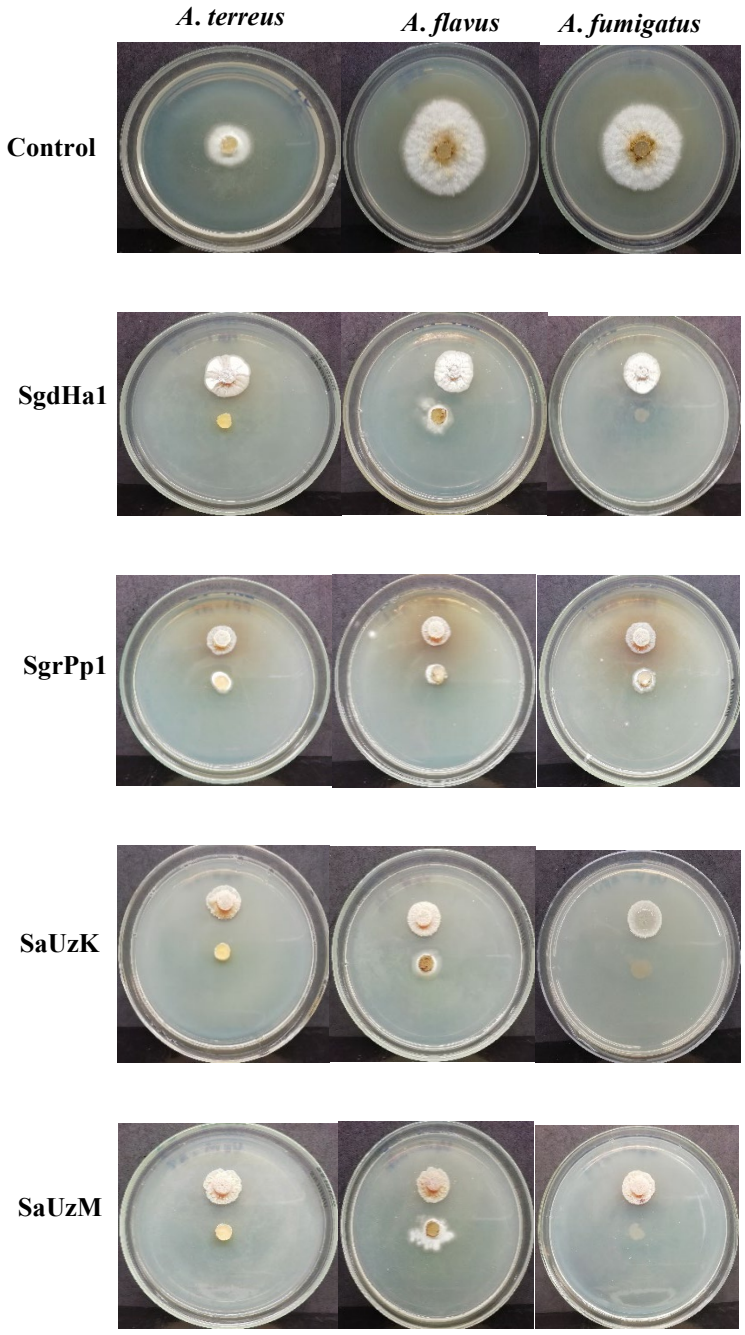

Figure 8: Anti-fungal activity of *S. gancidicus* isolate Ha1 (SgdHa1), *S. griseoruber* isolate Pp1 (SgrPp1) or *S. aureus* isolates UzK (SaUzK) and UzM (SaUzM) against *A. terreus*, *A. flavus* and *A. fumigatus* on 90 mm MHA plates. Control: fungal growth without actinobacteria

Table 2. Percentage of radial growth inhibition of *F. striatum* Rosa (FstR), *F. solani* Branco.branco (FsoBB), *Mucor fragilis* Cinza.cinza. (MfCC), *P. citrinum* Verde (PcV), *C. madrasense* Kala (CmK), *A. terreus* (AT), *A. fumigatus* (AFU) and *A. flavus* (AFL) by *Streptomyces* spp. *S. gancidicus* isolate Ha1 (SgdHa1), *S. griseoruber* isolate Pp1 (SgrPp1), *S. aureus* isolates UzK (SaUzK) or UzM (SaUzM).

Actinobacteria	Filamentous fungi								Average (%)
	FstR	FsoBB	MfCC	PcV	CmK	AT	AFL	AFU	
SgdHa1	40.20	36.04	75.25	100.00	34.16	100	68.70	100	69.29
SgcPp1	26.96	62.61	18.18	51.39	15.64	45.83	71.42	73.05	45.64
SeUzK	31.86	48.20	48.99	100.00	20.99	100	63.26	100	64.16
SeUzM	71.57	72.52	52.53	100.00	13.58	100	61.90	100	71.51
Average (%)	42.65	54.84	48.74	87.85	21.09	86.46	66.33	93.26	

Percentage inhibition of radial growth (Table 2) of the *F. striatum* Rosa isolate ranged from 26.96 to 71.57. *S. aureus* isolate UzM showed the highest inhibition (71.57), followed by *S. gancidicus* isolate Ha1 (40.20), *S. aureus* isolate UzK (31.86) and *S. griseoruber* isolate Pp1 (26.96).

Another species of FSSC, *F. solani* Branco.branco isolate was also mainly inhibited by the *S. aureus* isolate UzM (72.52%), followed by *S. aureus* isolate UzK (48.20%) and *S. gancidicus* isolate Ha1 (36.04%).

Radial growth inhibition of *M. fragilis* was 75.25% by *S. gancidicus* isolate Ha1, 52.53% by *S. aureus* UzM, 48.99% by *S. aureus* UzK, and 18.18% by *S. griseoruber* isolate Pp1.

Interestingly, all the *Streptomyces* spp. completely abolished the growth of *P. citrinum*, except for *S. griseoruber* Pp1 isolate, which inhibited growth by 51.39%.

The *C. madrasense* isolate Kala was more resistant to inhibition by *Streptomyces* spp., ranging from 13.58% (*S. aureus* UzM isolate) to 34.16% (*S. gancidicus* Ha1 isolate).

A. terreus was completely inhibited by *Streptomyces* spp., except by the *S. griseoruber* Pp1 isolate (45.83% inhibition). Growth inhibition of *A. flavus* was 71.42% by *S. griseoruber* isolate Pp1, 68.70% by *S. gancidicus* isolate Ha1, 63.26% by *S. aureus* UzK, and 61.90% by *S. aureus* isolate UzM (Table 2). *A. fumigatus* was also completely abolished by *Streptomyces* spp., except for *S. griseoruber* isolate Pp1 (73.04% inhibition).

Discussion

Worldwide, few studies on subterranean ferruginous regions fungi were conducted (VANDERWOLF et al., 2013; ZHANG et al., 2017b). There is a gap in our knowledge of this

mycobiome, especially in Brazil. Studies on this field shall contribute to the understanding of fungi diversity and to the biotechnological exploitation of these microorganisms.

Different *Penicillium* species were detected from Ferruginous Crusts and Stalactites located in the South-West of Romania in the Banat Mountains (GHERMAN et al., 2014).

TAYLOR and colleagues 2014) isolated 34 fungal species distributed among 12 genera from the Iron quadrangle (Quadrilátero Ferrífero), Nova Lima, Minas Gerais, Brazil. Among them, two species of *Fusarium*, 11 species of *Penicillium* and three species of *Mucor* were suggested as potential human opportunistic pathogens for the ability to grow at 37 °C.

Fourteen *Fusarium* sp. were reported from Dalli and Rajhara iron ore mines located in the state of Chhattisgarh, India (VERMA; SINGH; VERMA, 2016).

Held and colleagues (2020) explored the fungal diversity in Soudan iron mine of Lake Vermilion Soudan Underground Mine State Park in Tower Minnesota. Out of 164 identified taxa, 108 belong to the Ascomycota phylum, 26 to Basidiomycota and 31 to Mucoromycota (HELD; SALOMON; BLANCHETTE, 2020).

Mucoromycota were the most abundant fungi isolated from the mud collected one year after the collapse of two iron ore tailing dams in Mariana, Minas Gerais state, Brazil (GIONGO et al., 2020).

In our study, from the Carajás National Forest iron ore plateau, we identified the following fungi isolates: two belonging to *F. striatum* FSSC 21, two belonging to *F. solani* FSSC 24a, two *M. fragilis*, one *M. irregularis* and one *P. citrinum*. These organisms have already been proposed as opportunistic human pathogens (MORI et al., 1987; KRISHNAN et al., 2015; MOK et al., 1997; PATERSON; LIMA, 2017). The species above were able to grow at 37 °C (Figure 6), but other phenotype features must be addressed before speculating them as possible opportunist human pathogens.

Fusarium spp., *Mucor* spp., *Pencillium* and *Aspergilli* correspond to an important cause of mortality in immunocompromised people. In this view, the fungal isolates obtained in this study could be employed as model organisms for filamentous fungi virulence. We also evaluated the susceptibility of these fungi, of *C. madrasense* and of three *Aspergillus* spp. to inhibition by the actinobacteria isolates we previously demonstrated to present antifungal activity against the *Cryptococcus* and *Candida* pathogenic yeasts (Ranjan et al., manuscript in preparation).

In the confrontation studies (Figure 7 and Table 2), *F. striatum* isolate Rosa was significantly inhibited by S. aureus isolate UzM, followed by *S. gancidicus* isolate Ha1, *S. aureus* isolate UzK and *S. griseoruber* isolate Pp1. Considering *F. solani* isolate Branco.branco growth inhibition, *S. aureus* isolate UzM was the most effective, followed by *S. griseoruber* isolate Pp1, *S. aureus* isolate UzK and *S. gancidicus* isolate Ha1. These results are in agreement with previous studies which showed that *Streptomyces* spp. can be used as biocontrol agents against *Fusarium* spp. (ZAMOUM et al., 2015; KHEBIZI et al., 2017; HU et al., 2019; SÁNCHEZ-GARCÍA et al., 2019;).

Fusarium was the most prominent fungus in patients with keratitis/corneal ulcer at Aravind Eye Hospital, Coimbatore, India (MANIKANDAN et al., 2019). BATISTA and colleagues (2020) indicated fusariosis provoked by drug resistant isolates as an emerging fungal infection which is difficult to treat and also proposed alternative treatment regimens to face fungal resistance. DA ROSA and colleagues (2021) investigated the epidemiology of Fusarium spp. infections at the Hospital de Clinicals de Porto Alegre (HCPA), Rio Grande do Sul, Brazil. From 2008 to 2017, twenty-seven fusariosis patients were diagnosed. FSSC was identified from 76.2% patients. Thirty-one percent of the FSSC clinical isolates were amphotericin B resistant. FSSC clinical isolates also showed resistance to voriconazole.

The *S. gancidicus* Ha1 isolate showed the strongest antifungal activity against *M. fragilis* Cinza.cinza, followed by *S. aureus* isolates UzM and UzK, and *S. griseoruber* isolate Pp1 (Figure 7 and Table 2).

Studies on *M. fragilis* as a human pathogen are rare. YANG and collaborators (2016) identified *M. fragilis* from Mucorales isolates collected during 2010-2014 from the Samsung Medical Center, Seoul, Korea.

There is no study on inhibition of *M. fragilis* by *Streptomyces*. Though, SRIPREECHASAK and collaborators (2013) reported that six *Streptomyces* spp. isolates, purified from the soil of the Krung Ching Waterfall, Nakhon Si Thammarat province (Thailand) were antagonist to *M. racemosus*. Some other studies also reported the antagonistic activity of *Streptomyces* against *M. racemosus* (MATSUMOTO; TAKAHASHI, 2017; CHAROUSOVÁ et al., 2019).

Recently, mucormycoses were declared epidemic and notifiable diseases in several states of India. During December, 2019 to April 2021, 71% of global mucormycosis cases were reported from India (JOHN; JACOB; KONTOYIANNIS, 2021). Due to the second wave of COVID-19, more than 15000 cases were reported from India as of May 28, 2021, state of Gujarat contributed 3726 cases followed by Maharashtra (RAUT; HUY, 2021). In this view, the identification of new compounds capable of controlling Mucorales growth are extremely important.

In our study, *S. gancidicus* isolate Ha1, and *S. aureus* UzK and UzM isolates completely abolished the growth of *P. citrinum* (Figure 7 and Table 2). *P. citrinum* growth inhibition by *S. lacticiproducens* GIMN4.001, isolated from the rhizosphere of tomato plant in China, was reported by ZHU et al. (2011). ZHANG and colleagues (2017a) reported the inhibition of *P.*

citrinum growth by the extract of *Streptomyces* TRM 49605, isolated from desert soil, also in China.

HESSE and co-workers (2017) reported, for the first time, *P. citrinum* cross-reactivity with a clinically validated assay for the detection of *Aspergillus* galactomannan. The former was isolated from the bronchoalveolar lavage of a patient. Minimum inhibitory concentrations (μg mL^{-1}) reported for this strain were 1.00 for amphotericin B; 0.125 for caspofungin; 0.06 for micafungin; 4.00 for itraconazole; 2.00 for posaconazole and >16 for voriconazole. This illustrates the relevance of studying new antifungal substances against *P. citrinum.*

In our study, *C. madrsense* isolate Kala was significantly inhibited by *S. gancidicus* isolate Ha1, followed by, *S. aureus* isolate UzK, *S. griseoruber* isolate Pp1, and *S. aureus* isolate UzM. (Figure 7 and Table 2). Our study is the first one to report *C. madrasense* inhibition by *Streptomyces* spp.

Chaetomium species are globally found in soil and cellulosic materials. *Chaetomium* spp. has the ability to grow at high temperatures, which allows them to survive in host conditions and cause opportunistic infections (AHMED et al., 2016). They may cause superficial and deep mycoses, brain abscess, sinusitis, pneumonia, allergic reactions and invasive pulmonary infections (CAPOOR et al., 2016; GABRIO; WEIDNER, 2018).

Aspergillus fumigatus is the most frequent human pathogen among *Aspergillus* spp. (67–73%), followed by *A. flavus* (10–16%), *A. niger* (5–9%) and *A. terreus* (3–4%) (PERFECT et al., 2001; STEINBACH et al., 2012; CHAKRABARTI et al., 2019). In our work, *S. gancidicus* isolate Ha1, and S. aureus UzK and UzM isolates completely inhibited the radial growth of *A. terreus* and *A. fumigatus*. *S. griseoruber* isolate Pp1 showed the strongest inhibitory activity against *A. flavus*, followed by *S. gancidicus* isolate Ha1, *S. aureus* isolates

UzK and UzM (Figure 8 and Table 2). These data are promising, since *Aspergillus* spp. infections present a major danger to human health, presenting a high mortality rate.

Drug-resistant *Aspergilli* isolates description is increasing and poses a huge threat to human beings. One of the main reasons of the selection of drug resistant isolates is the application of fungicides in agriculture. CHEN and colleagus (2018) conducted a study on azole resistant *A. fumigatus* isolates from agricultural fields in China. Out of 206 isolates, 10.2% were resistant to azoles due to mutations in the Cryp51A gene.

BABANAGARE and VIDYASAGAR (2011) reported the antifungal activity of *Streptomyces* JF714876 against *A. terreus*, by deforming and distorting the mycelial structure.

Several studies reported the antifungal activity of *Streptomyces* against *A. flavus* and A. *fumigatus*. *S. roseolus*, isolated from soil in Algeria, reduced the production of aflatoxin B1 and kojic acid by A. flavus by downregulating the aflatoxin gene cluster (AFB1) and the kojR gene (CACERES et al., 2018). Volatile organic compounds (VOCs) released by *S. alboflavus* TD-1, isolated from China, delayed the *A. flavus* mycelial growth (Yang et al., 2019). Fifty-eight Streptomyces isolates from soil, in France, inhibited the *A. flavus* growth and reduced the aflatoxin production (CAMPOS-AVELAR et al., 2021).

Streptomyces sp. VITSVK5, isolated from marine sediments of the Bay of Bengal, India, inhibited the growth of *A. fumigatus* ATCC 6645. The same isolate also inhibited the growth of multidrug resistant clinical *Aspergillus* isolates obtained from sputum of pulmonary tuberculosis patients admitted to the Government Vellore Medical College and Hospital (GVMCH), Tamil Nadu, India (KUMAR; KANNABIRAN, 2010). HADIZADEH et al., (2015) demonstrated the antifungal activity of *S. rochei*, isolated from soil in Iran, against an azole-resistant *A. fumigatus* isolate. *S. libani*, isolated from the same source, inhibited the

mycelial growth and sporulation of *A. fumigatus* (AZISH; SHAMS-GHAHFAROKHI; RAZZAGHI-ABYANEH, 2020).

Our data clearly indicated the inhibitory potential of *Streptomyces* spp. isolated from rhizosphere soil of Indian medicinal plants against diverse potential opportunist pathogenic filamentous fungi. In this view, the fractionation of the crude extracts and the screening for the bioactive compounds shall result in the identification of new antifungal metabolites.

Conclusion

In the present study, we describe the isolation of *Fusarium striatum*, *F. solani*, *M. fragilis* and *P. citrinum* from the Carajás National Forest for the first time. These fungi, as well as *C. madrasense*, *A. terreus*, *A. flavus* and *A. fumigatus* were inhibited upon co-culture with *S. gancidicus*, *S. griseoruber* and *S. aureus*. Our data can contribute to the studies on the mycota diversity from iron ore mining areas and to the identification of new antifungal compounds from actinobacteria.

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Supplementary Figures

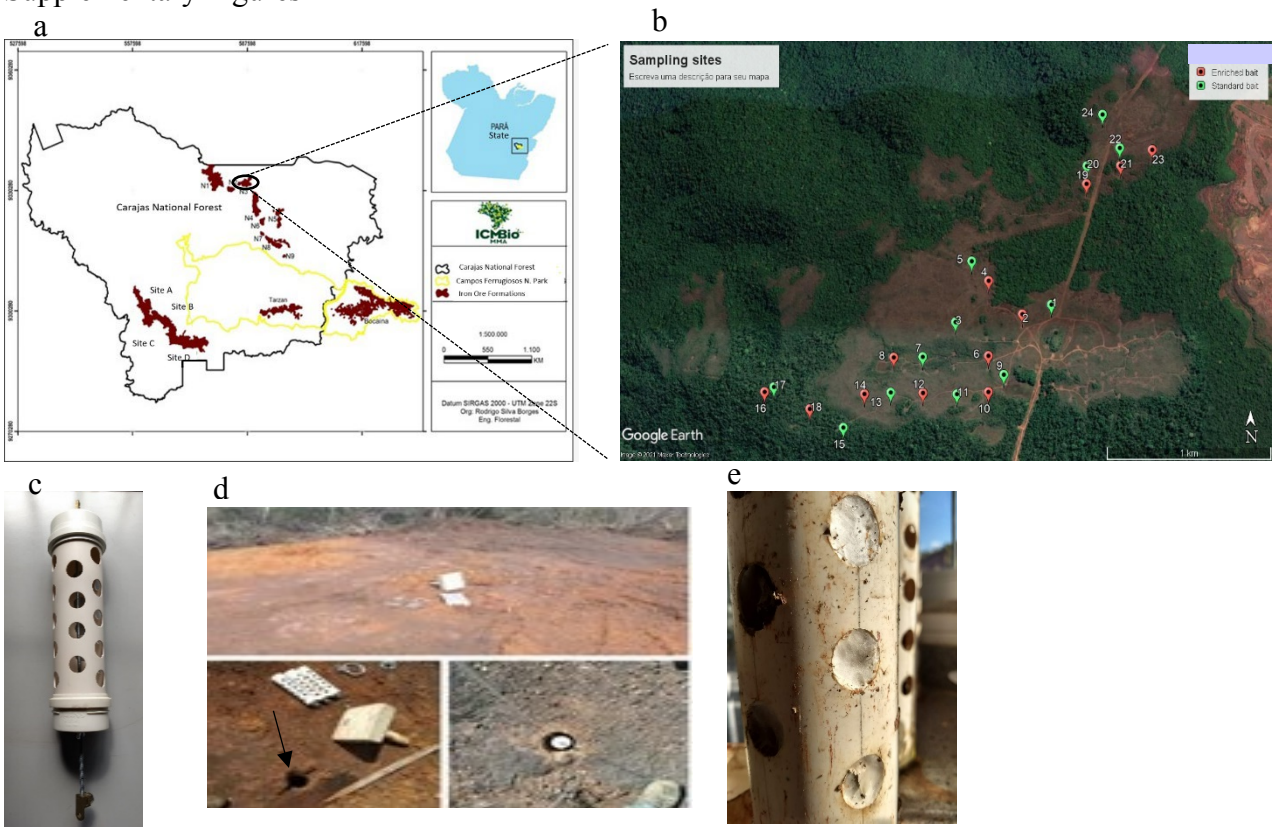


Figure S1: Map of the Carajás National Forest the iron ore plateau areas in brown (a) Source: ICMBio. The N3 plateau of this region (b) was selected for the study; the green dot represents traps with standard bait and red with enriched bait. Traps (c) were inserted in the bore hole (indicated by black arrow) (d). After some 8 weeks, traps were removed and some of them were blocked by filamentous, fungal-like material (e).

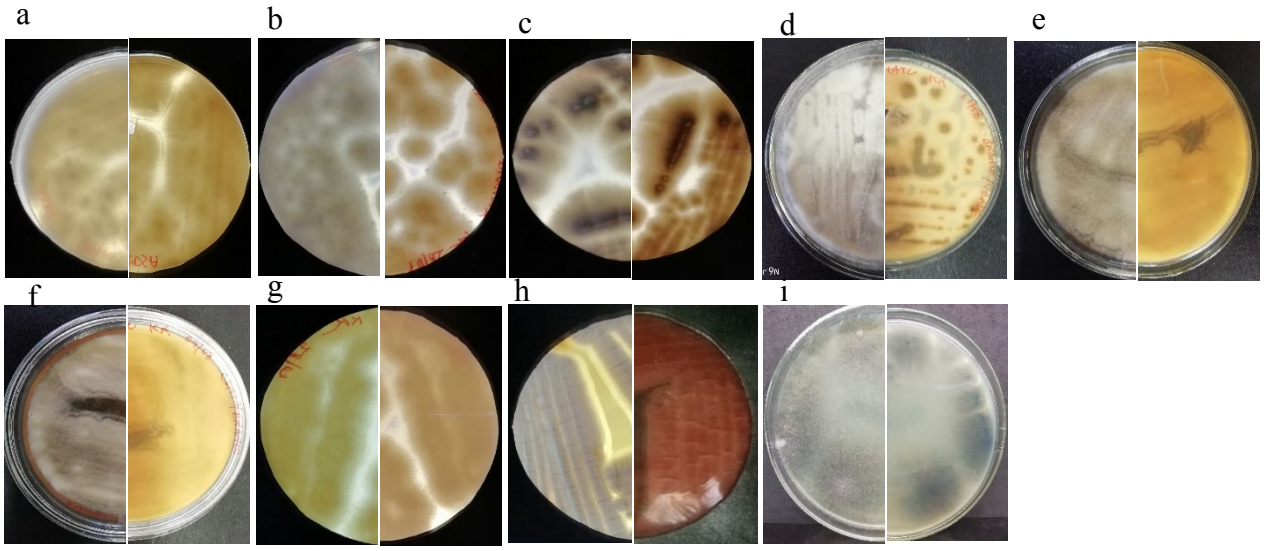
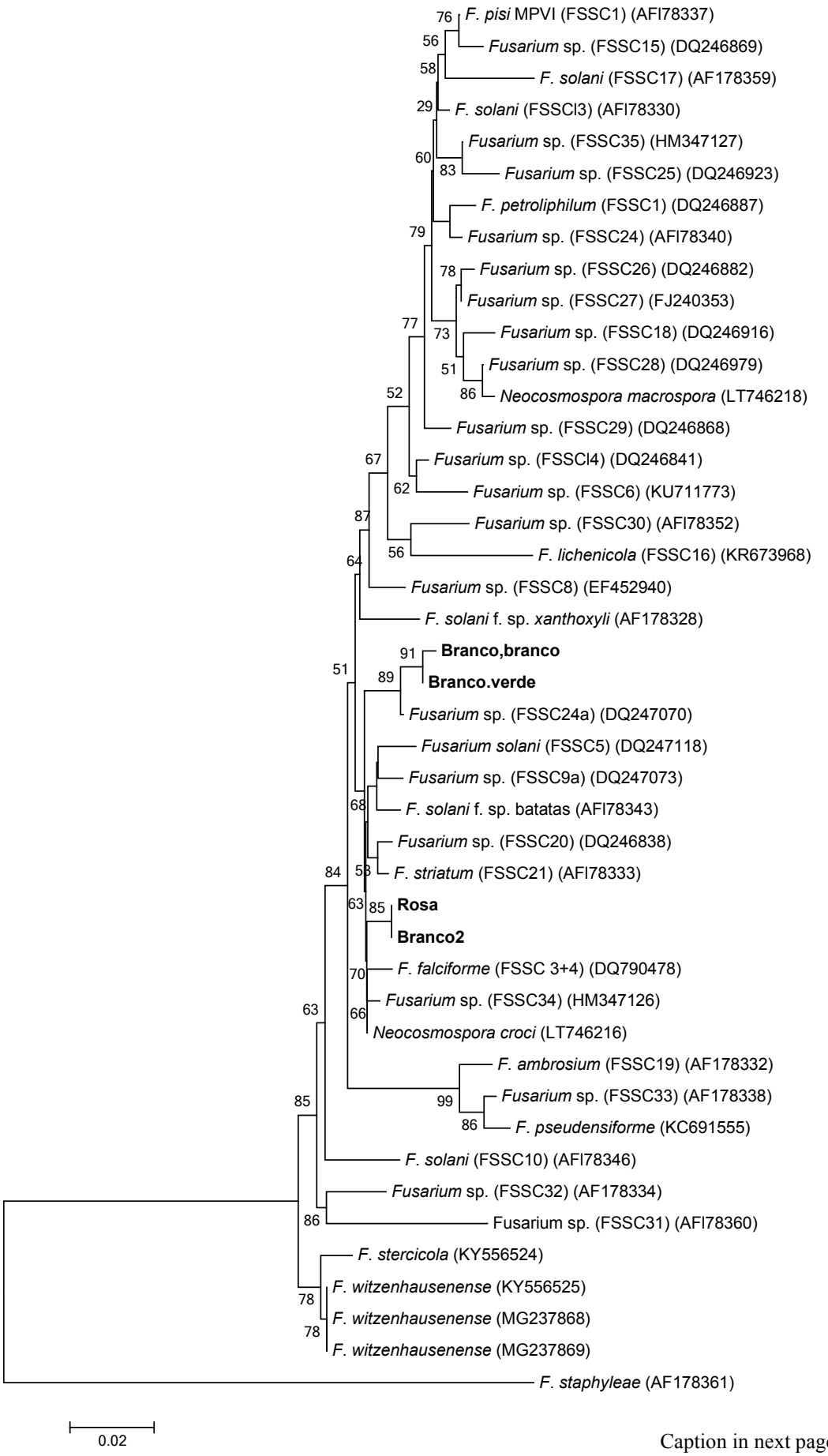


Figure S2: Front and reverse side photographs of fungi grown on 90 mm YPD plates, at 28 °C, for 7 days. a) Rosa, b) Branco2, c) Branco.branco, d) Branco.verde, e) Cinza.cinza, f) Cinza.branco, g) Amarelo, h) Verde and i) Kala



Caption in next page

Figure S3: Phylogram showing the position of Branco.branco, Branco.verde, Rosa and Branco2 in relation to other *Fusarium solani* species complex (FSSC) based on *tef1* gene sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Fungi isolates from this study are in bold. Accession numbers are represented in parenthesis

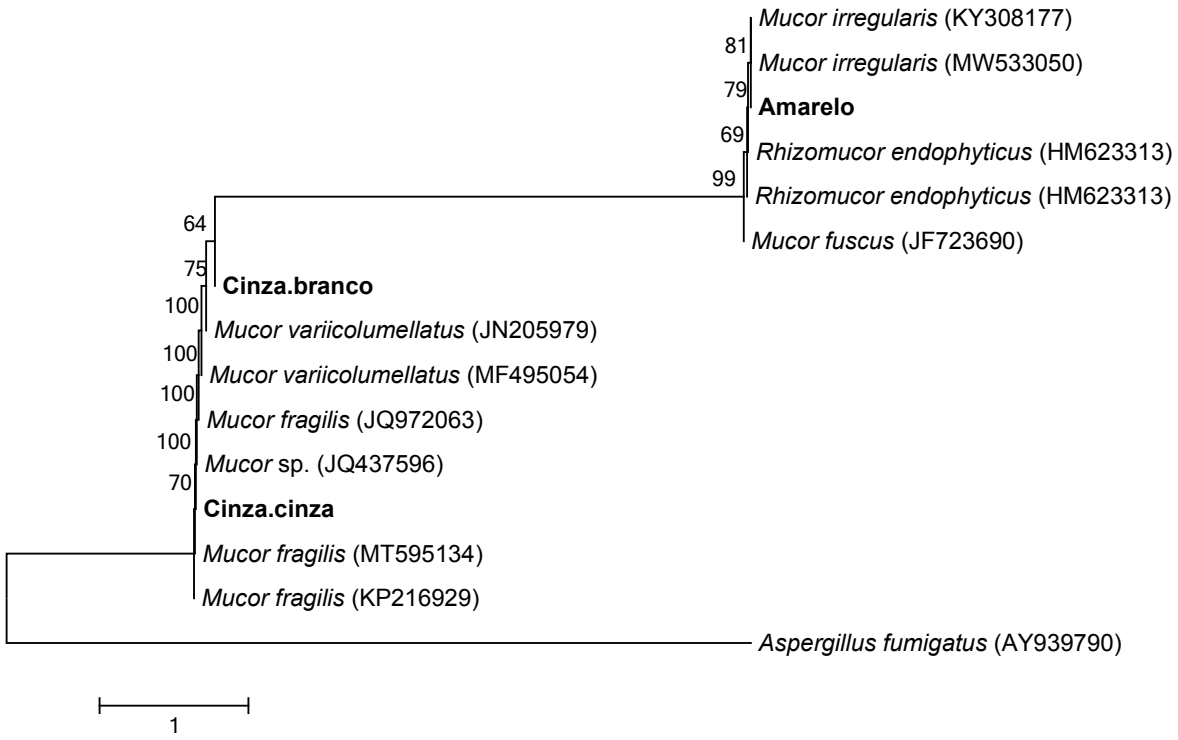


Figure S4: Phylogram showing the position of Amarelo, Cinza.branco and Cinza.cinza in relation to other mucor spp. based on *Mucor* specific ITS sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Fungi isolates from this study are in bold. Accession numbers are represented in parenthesis

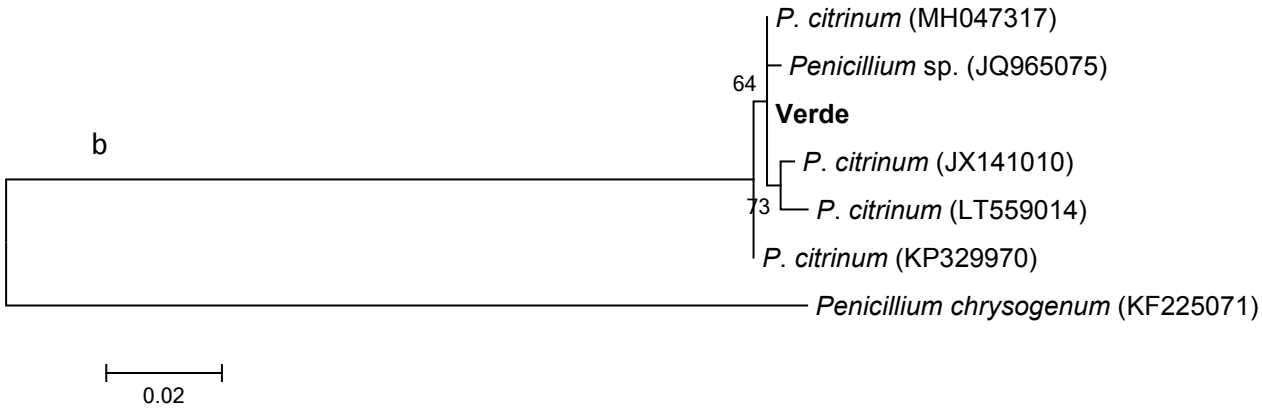


Figure S5: Phylogram showing the position of Verde in relation to other *Penicillium* spp. based on *benA* gene sequences. The tree is constructed based on maximum likelihood analysis. The values next to the nodes indicate bootstrap support (%) based on the analysis of 1000 replicates. Verde appears bold. Accession numbers are represented in parenthesis.

Supplementary Tables

Table S1: Primer sets used in this study

Primers Forward/ Reverse	Target gene	Amplicon Length (bp)	Thermal Cycling profile (Initial denaturation 95 °C for 3 mins and final extension 72 °C for 10 mins)	References
ITS1/ ITS4	Fungal ITS	550	30 cycles of 95 °C for 30 s; 55 °C for 60 s; 72 °C for 60 s	WHITE et al., 1990
Oli Mucor1/ Oli Mucor4	*Mucor* specific ITS	263	30 cycles of 95 °C for 30 s; 50 °C for 30 s; 72 °C for 30 s	BERNAL-MARTÍNEZ et al., 2013
TEF-Fs4f / TEF-Fs4ra	*Fusarium* translation elongation factor (*tef1)*	650	35 cycles of 95 °C for 30 s; 65 °C for 30 s; 72 °C for 30 s	MOINE et al., 2014
Bt2a/ Bt2b	*Penicillium* specific *Beta*-tubulin (*benA*)	550	35 cycles of 95 °C for 45 s; 55 °C for 45 s; 72 °C for 60 s	MIDORIKAWA et al., 2014

4. CONCLUSION

The purpose of this work was to evaluate different strategies to control the growth of *Cryptococcus* and to address the role of *C. neoformans* histone deacetylase genes in the response to antifungal drugs, epigenetic modulators and photodynamic therapy.

We demonstrated the synergy of antifungal drugs and epigenetic modulators only for histone deacetylase gene deletion mutants in *C. neoformans*. Therefore, we speculated that HDAC impairment with enzyme inhibitors, or by gene deletion, is related to increased drug susceptibility, particularly to fluconazole.

Amphotericin B, fluconazole, sodium butyrate (HDACi), or hydralazine (DNMTi), potentiated the antifungal activity of photodynamic therapy (PDT) against the HDAC gene deletion strains. An interplay between the HADC activity and the response to PDT-induced oxidative stress was proposed. In this view, the combination of antifungals, epigenetic drugs and PDT could represent a less invasive alternative therapy for the cutaneous manifestation of *Cryptococcus* infections.

Drug resistant pathogens threaten our ability to treat common infections globally. The World Health Organization declared antimicrobial resistance one of the top 10 global public health threats. Drug resistant *Cryptococcus* isolates contribute to the treatment failures. New antifungals, such as compounds obtained from actinobacteria, are in urgent need. With this in mind, we also evaluated the antifungal activity of the crude extracts of new *Streptomyces* isolates against *Cryptococcus* spp. The *S. griseoruber* Pp1 crude extract presented increased inhibition of fluconazole-resistant *Cryptococcus* strains growth, in comparison to the H99 reference strain. The crude extract of *S. aureus* UzM was the most effective in inhibiting *C. gattii*.

A pronounced effect of the actinobacteria crude extracts was verified against HDAC gene deletion mutant strains (*hda1*Δ, *hos2*Δ, *hda1*Δ/*hos2*Δ and *hda1*Δ+ HDA1). This could be related to an additive chromatin remodelling impairment and/or to HDAC/DNMT enzymes inhibition.

In another chapter of our work, we addressed the mycota from the subterranean iron ore plateau in the Carajás National Forest (Brazil). We obtained *F. striatum*, *F. solani*, *Mucor irregularis*, *M. fragilis*, *P. citrinum* isolates, described for the first time from this region. Since filamentous fungi from the *Fusarium* and *Mucor* genera can cause opportunistic infections in humans, and multi-drug resistant strains pose a threat to clinics worldwide, we wondered whether the species we obtained could be employed as model organisms for the study on the antifungal activity of actinobacteria.

We verified that the *Streptomyces* isolates we purified from the rhizosphere of Indian Traditional Medicine plants presented antifungal activity against diverse filamentous fungi which could represent opportunistic pathogens to humans (*F. striatum*, *F. solani*, *M. fragilis*, *P. citrinum*, *C. madrasense*, *A. terreus*, *A. flavus* and *A. fumigatus*)

5. PERSPECTIVES

The results of this work pave the way for more in-depth evaluation of the mechanisms of interaction among antifungal drugs, epigenetic modulators and PDT, aiming not only the understanding of biological processes, but also the design of alternative therapeutic regimens for fungal infections. The screening of new actinobacteria isolates presenting antifungal activity can contribute to the discovery of new antimicrobial metabolites. In this view, future experiments shall focus:

- The evaluation of combined exposure of antifungals, epigenetic drugs and PDT in other important pathogenic fungi which afflict developing countries, such as *Candida* spp., *Paracocidioides* spp., *Sporotrix* spp. and *Mucor* spp.
- The evaluation of the combined therapy on fungal virulence traits, such as, melanin synthesis, phospholipase, proteases and ureases production, mating ability, morphological transition and biofilm formation.
- Studies of the combined therapy on animal models for superficial fungal infections.
- The purification and structural characterization of actinobacteria bioactive compounds showing antifungal activity.
- The purification and structural characterization of new chromatin states modifiers from actinobacteria.
- The evaluation of the synthesis, by the actinobacteria isolates, of eco-friendly nanoparticles which could be employed for therapeutics and/or the design of drug delivery system

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