

In Vivo Pathogenicity Characterization of Viable Opportunistic Fungi *Aspergillus thermomutatus* and *Rhodotorula mucilaginosa* Recovered from Maritime Antarctic Permafrost

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Cite This: <https://doi.org/10.1021/envhealth.4c00213>

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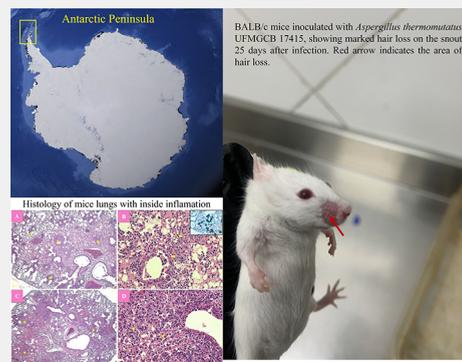
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ABSTRACT: In this study, we evaluated the pathogenic potential of the fungi *Aspergillus thermomutatus* and *Rhodotorula mucilaginosa* obtained from maritime Antarctic permafrost using *in vivo* experiments on immunocompromised BALB/c mice. Despite the low mortality observed, immunosuppressed animals infected with *A. thermomutatus* and *R. mucilaginosa* exhibited fluctuations in body mass and induced changes in the neuropsychiatric state of the mice. Fungi were recovered from the lungs, spleen, blood, and brain of infected mice at densities similar to but slightly lower than the inoculum up to 5 days post-inoculation. *A. thermomutatus* infection induced an inflammatory process in the lungs of infected BALB/c mice. In the target organs of animals infected with *R. mucilaginosa*, a notable fungal load was detected in the brains of infected animals. These results suggest that viable isolates of fungi such as *A. thermomutatus* and *R. mucilaginosa* originating from Antarctic permafrost, which is exposed to increasing melt caused by rising temperatures in the region, may present significant pathogenic potential. This highlights that climate change in Antarctica may facilitate the release and dispersal of fungi and other pathogenic microorganisms capable of infecting humans and animals.

KEYWORDS: Antarctica, climate change, extremophiles, virulence, fungi



1. INTRODUCTION

Among the global fungal diversity (~105000 species known), those classified as extremophiles have been studied in various contexts, including their potential as pathogens. However, until now, few studies have reported the isolation of fungi with pathogenic potential toward humans, animals, and plants from substrates collected in Antarctica,^{1–4} and little is yet known about the pathogenic characteristics of these fungi.

The genus *Aspergillus* includes species frequently reported from various habitats, including Antarctic substrates, and includes cosmopolitan species adapted to the extreme conditions of the continent.^{5,6} Of the ~250 species of *Aspergillus* known, 16% are considered to be opportunistic pathogens of humans and immunocompromised animals.⁷ *Aspergillus thermomutatus* (teleomorph *Neosartorya pseudofischeri*) is among the *Aspergillus* species in the section *Fumigati* that is recognized as pathogenic⁸ and is reported to cause aspergillosis, peritonitis, and other diseases.^{9–13} Recent studies have also shown that members of *Aspergillus* are involved in fungal co-infection in COVID-19 patients.^{14–16}

Members of the yeast genus *Rhodotorula* have also been detected in different Antarctic substrates.^{1,2,6,17} Three species in

this genus have been implicated in immunocompromised patients, *R. mucilaginosa*, *R. glutinis*, and *R. minuta*.¹⁸ *Rhodotorula mucilaginosa* occurs in marine, aquatic, and terrestrial environments globally, colonizing many substrates.¹⁹

Parts of Antarctica currently face accelerated rates of climate change, particularly increasing temperatures, relative to global averages,²⁰ one consequence of which is to increase the rate of melting of permafrost. This phenomenon has been suggested to potentially contribute to the release and dispersal of pathogenic fungal propagules within Antarctica and to other southern land masses.^{2–5}

There is currently a general lack of knowledge of the pathogenicity of the Antarctic fungal community. da Silva et al.⁶ reported a diversity of viable fungi present in samples of Antarctic permafrost, including strains of *A. thermomutatus* and

Received: October 12, 2024

Revised: January 17, 2025

Accepted: January 22, 2025

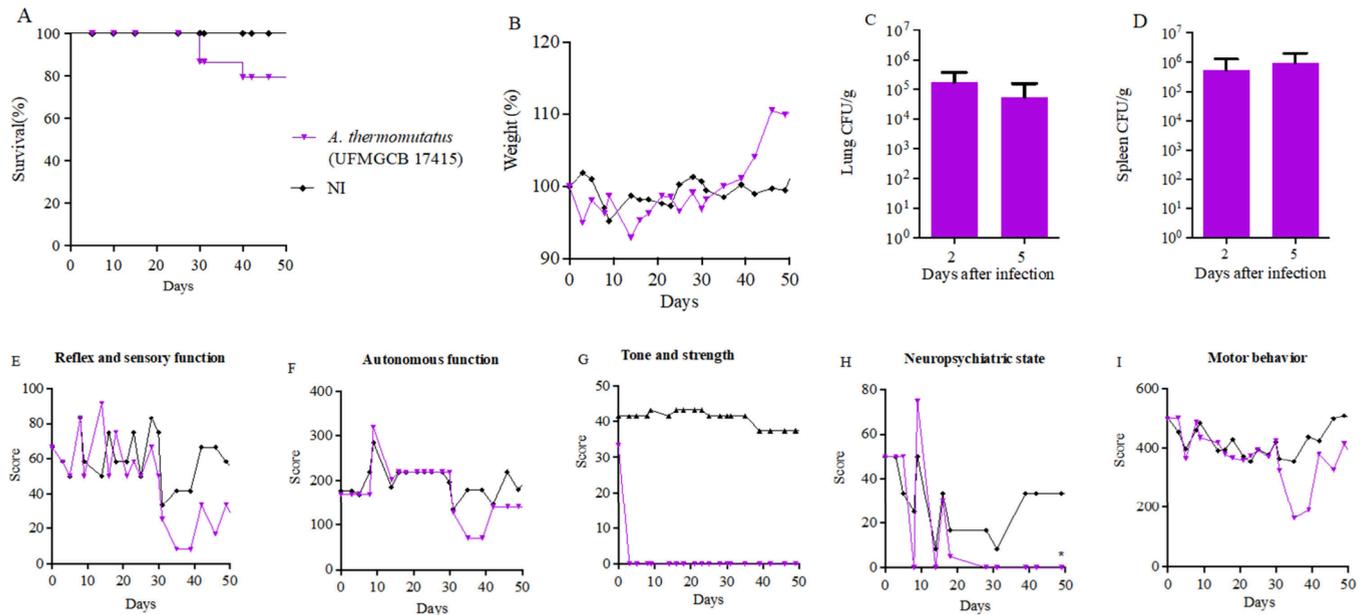


Figure 1. Survival and behavior of immunosuppressed BALB/c mice after infection with spores of *Aspergillus thermomutatus* UFMGCB 17415. (A) Survival of mice infected intranasally with 1×10^8 CFU mL⁻¹ ($n = 6$ mice per group), (B) mouse body mass variation (%) over time after infection, fungal load recovered from the (C) lung and (D) spleen 2 and 5 days after infection, (E) sensory function and reflex, (F) autonomous function, (G, H) neuropsychiatric status, and (I) motor behavior. (NI) Non-infected control. (*) Statistically significant difference relative to NI ($p < 0.05$).

R. mucilaginosa, which displayed pathogenic potential and capability to kill larvae of *Tenebrio molitor*. In the current study, we further evaluated the pathogenicity of these permafrost-sourced fungal strains against immunocompromised BALB/c mice.

2. MATERIALS AND METHODS

2.1. Fungal Origin

Three fungal strains previously isolated from permafrost samples were used in this study. *Aspergillus thermomutatus* UFMGCB 17415 was originally obtained from the Keller Peninsula and *R. mucilaginosa* UFMGCB 17448 from Thomas Point (both on King George Island) and *R. mucilaginosa* UFMGCB 17473 from Whalers Bay (Deception Island). All three locations are in the South Shetland Islands, maritime Antarctica. All three strains can grow at 37 °C on different media.⁶ The strains are deposited in the Collection of Microorganisms and Cells of the Universidade Federal de Minas Gerais, Brazil, and were previously characterized by da Silva et al.⁶

2.2. Fungal Inocula

Aspergillus thermomutatus UFMGCB 17415 was cultured on potato dextrose agar (PDA) at 30 °C for 7 days. The yeast strains *R. mucilaginosa* UFMGCB 17448 and UFMGCB 17473 were cultured under the same conditions for 3 days. After the initial 7 or 3 days culture, the *A. thermomutatus* mycelia were covered with sterilized saline solution (0.85% NaCl) while batches of both *R. mucilaginosa* isolates were added to sterilized saline solution (0.85% NaCl). In each case, the liquid containing spores from the filamentous fungus and yeast cells was transferred to a sterilized test tube. After sedimentation and decanting, the supernatant was vortexed for approximately 15 s and stained with Trypan Blue for viability detection and counting in a Neubauer chamber. Inocula of 1×10^8 mL⁻¹ viable spores of *A. thermomutatus* UFMGCB 17415 and 1×10^6 mL⁻¹ viable cells of *R. mucilaginosa* UFMGCB 17473 and UFMGCB 17448 were used in subsequent mouse infection assays.

2.3. Animals

In vivo assays followed the protocols established by Gomes et al.²¹ Female BALB/c mice ($n = 6$ mice per group), 6–8 weeks old, were obtained from the Universidade Federal de Minas Gerais for use in the

experiments. Procedures followed the Brazilian Society of Zootecnics/Brazilian College of Animal Experimentation guidelines and Federal Law 11794 and were approved by the Ethics Committee on Animal Use of the Universidade Federal de Minas Gerais (CEUA/UFMG, protocol no. 313/2016).

The mice were immunosuppressed using oral Dexamethasone (Decadron/Aché 10 mg kg⁻¹ day⁻¹) in their drinking water for 5 days prior to the experiment.²² A control group ($n = 6$ mice) was inoculated with phosphate buffered saline (PBS). After immunosuppression, groups of animals were inoculated intranasally with a suspension of fungal inoculum.

2.4. Mice Survival and Behavior and Fungal Burden

After immunosuppression and subsequent infection, the animals were monitored daily for survival, weight loss, and behavior using the SmithKline/Harwell/ImperialCollege/RoyalHospital/Phenotype Assessment (SHIRPA) protocol.^{23–26} The SHIRPA protocol followed the criteria established by Oliveira et al.²⁷ Fungal burden was assessed following Gomes et al.²¹ Results were expressed as CFU g⁻¹.

2.5. Recovery and Identification of Fungi Present in the Organs of Infected Immunocompromised BALB/c Mice

After analyzing the survival curve, euthanasia times were established to evaluate fungal load and allow histopathological analysis. New groups of uninfected (inoculated with sterilized 1× PBS) and infected animals, each consisting of four animals, were then euthanized after 2 and 5 days. Before euthanizing, the animals were anesthetized with a solution of ketamine (80 mg kg⁻¹; Syntec, Brazil) and xylazine (10 mg kg⁻¹; Ceva, Brazil), as established in protocol CEUA 172/2020. After total loss of reflexes, the animals were euthanized. Target organs were excised and prepared immediately. For intranasal infection, the spleen and lungs were evaluated, while for intravenous infection, the brain, spleen and blood were assessed. Using a manual homogenizer, cells were disrupted by maceration in 1 mL of PBS and subjected to a 10⁻² dilution of the suspension extracted from the organs and blood. One hundred microlitres of the suspension and dilutions of 1×10^{-1} mL⁻¹ and 1×10^{-2} mL⁻¹ were plated on Petri dishes containing PDA and incubated at 30 °C for 2 days for all strains. The fungal load was expressed in CFU g⁻¹ of tissue or per mL of blood.

The *A. thermomutatus* colonies recovered from the mice tissue were confirmed using molecular methods, following Rosa et al.²⁸ and

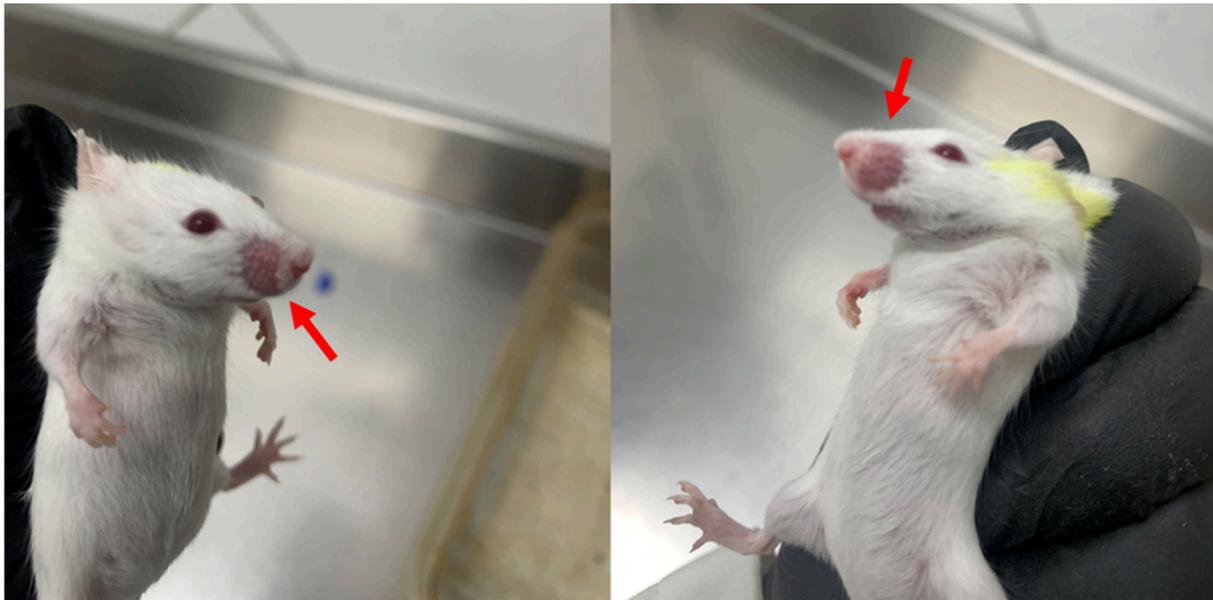


Figure 2. BALB/c mice inoculated with *Aspergillus thermomutatus* UFMGCB 17415, showing marked hair loss on the snout 25 days after infection. Red arrow indicates the area of hair loss. Photograph courtesy of E.C.Q.G.

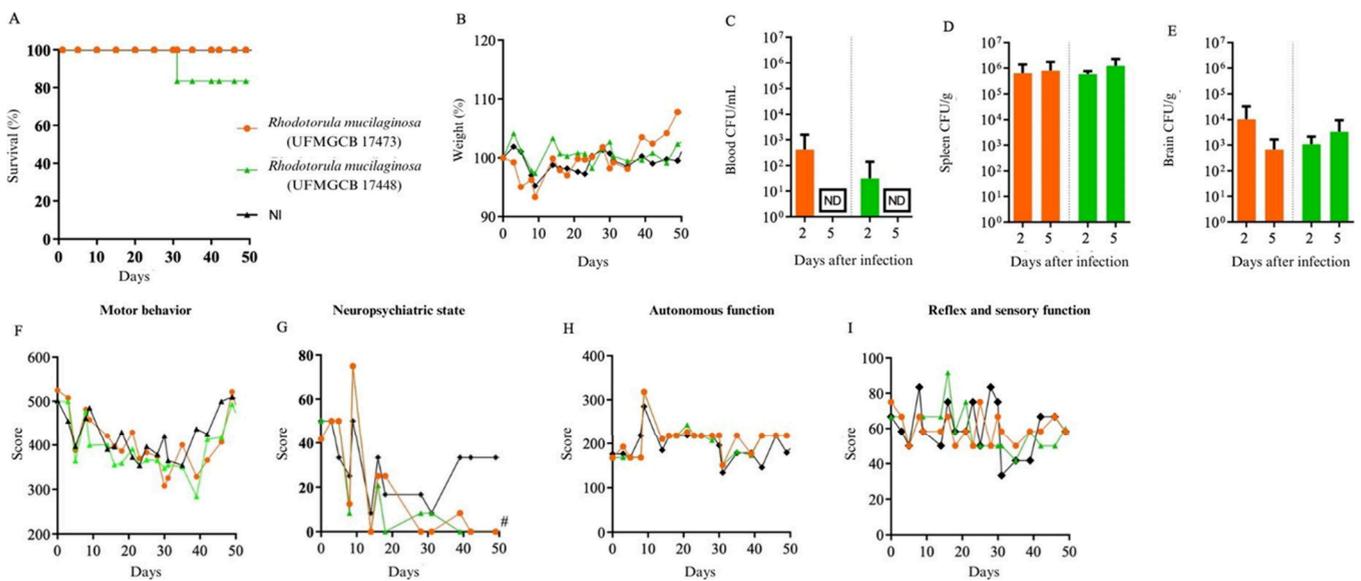


Figure 3. Survival, colony forming units (CFU), and behavioral characteristics of BALB/c mice after infection with *Rhodotorula mucilaginosa* isolates UFMGCB 174473 and UFMGCB 17448. (A) Survival curve of mice infected intravenously with 1×10^6 CFU mL^{-1} , (B) body mass variation of mice after infection expressed as a percentage (%), (C) fungal load recovered from blood, (D) spleen, and (E) brain 2 and 5 days after infection, (F) motor behavior, (G) neuropsychiatric status, (H) autonomous function, and (I) sensory function and reflex. (NI) Non-infected control. (#) Statistically significant difference relative to NI ($p < 0.05$).

banding patterns of microsatellite regions amplified via fingerprinting (PCR-MST) using the oligonucleotide (GTG)₅.²⁹ *Rhodotorula mucilaginosa* colonies recovered from the tissue were confirmed by morphological assessment.

2.6. Histopathology

The histopathological analysis followed the protocol of Gomes et al.²¹ Briefly, lungs, brain, and spleen, obtained 2 and 5 days post-infection (d.p.i.), were fixed by immersion in 10% buffered formalin for 24 h and embedded in paraffin. Sections of 4 μm thickness of the tissues were stained with hematoxylin and eosin. The type of cells involved in the inflammatory process and their extension and adjacent structures involved were described. The lesion score was assessed using the following scale: 0 = absence, 1 = discrete, 2 = moderate, and 3 = intense

inflammation. The presence of intralésional agents was assessed as absence (0) or presence (1).²¹

2.7. Statistical Analyses

Statistical analyses were carried out using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) with $p < 0.05$ considered significant. Kaplan–survival curves were prepared and analyzed according to the log rank test. For histopathological analyses, ANOVA was used. All experiments were performed at least twice.

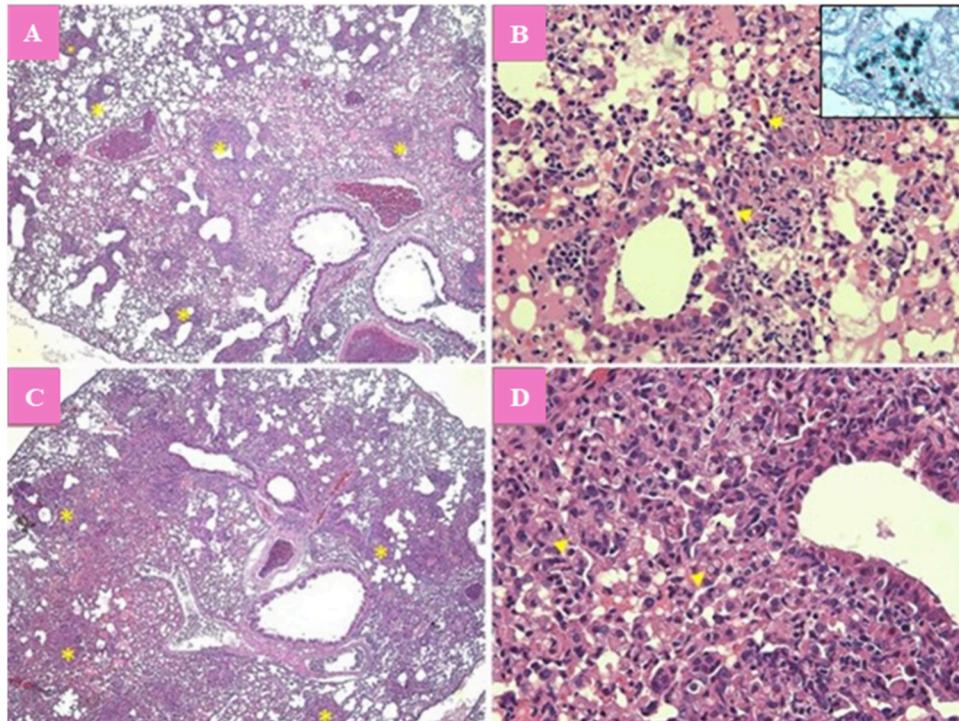


Figure 4. Lung tissue of BALB/c mice 2 days post-infection with *Aspergillus thermomutatus* UFMGCB 17415. (A) Multifocal areas of intense inflammatory infiltrate (*), located mainly around bronchioles. Hematoxylin–eosin (HE) staining, 5 \times objective. (B) Inflammatory infiltrate (*) with a large number of neutrophils and moderate number of epithelioid macrophages associated with structures measuring approximately 5 μ m with a thick capsule, characterized by a clear halo (not stained) surrounding the nucleus, which sometimes presented amphiphilic staining in the cytoplasm of macrophages (yellow arrowhead), in addition to moderate pulmonary edema and intense hyperemia. HE, 40 \times objective. The black fungal structures can be observed in detail. Grocott's Silver Methenamine Staining 100 \times objective. (C) Animals evaluated 5 days post-infection demonstrating multifocal areas of intense inflammatory infiltrate (*). HE, 5 \times objective. (D) Inflammatory infiltrate (*) composed of a large number of neutrophils and epithelioid macrophages, associated with a number of structures measuring approximately 5 μ m, thick capsule, characterized by a clear halo (not stained) surrounding the nucleus, which sometimes presented an amphiphilic color, in the cytoplasm of macrophages (arrowhead), in addition to moderate pulmonary edema and intense hyperemia. HE, 40 \times objective.

3. RESULTS

3.1. Survival, Fungal Burden, and Behavior Analysis

To evaluate the infection response of the fungi studied in an vivo model, immunosuppressed mice were infected with 10^8 spores of *A. thermomutatus* and survival curves were constructed (Figure 1A). Despite the low mortality level (20%) observed 35 days after infection with *A. thermomutatus*, the animals exhibited fluctuations in their body mass (Figure 1B) and the fungus was detected in their spleen and lungs at slightly lower levels than that of the original inoculum (Figure 1C,D).

In the assessment of sensory reflex function (Figure 1E), there was a significant change in scores for the infected groups 30 days after infection. For autonomic function (Figure 1F), scores were initially close to those of the control group but, 30 days after infection, the infected groups exhibited a decline followed by a recovery after 40 days. Neuropsychiatric state (Figure 1G,H) and motor behavior (Figure 1I) scores in the groups of infected animals showed a reduction compared to the control group from day 18 onward with this being significant for neuropsychiatric state. At 25 days after infection, the mice began to lose hair on their snouts, a characteristic known as shaving (Figure 2).

To generate survival curves after infection with the yeast isolates *R. mucilaginosa* UFMGCB 10448 and 17473, previously immunosuppressed animals were infected. Mortality of 20% was again observed 30 days after infection with *R. mucilaginosa* isolate UFMGCB 17448 (Figure 3A). Body mass oscillations

were observed in groups infected with both *R. mucilaginosa* strains (Figure 3B). The initial inoculum and recovered fungal loads were similar in the blood (Figure 3C), spleen (Figure 3D), and brain (Figure 3E) of infected animals.

Although there was variation in motor behavior (Figure 3F) between the yeast-infected groups of mice and the uninfected control group, by the end of the study period the scores were similar across all groups. The neuropsychiatric state (Figure 3G) also showed fluctuations, but at the end of the study period the yeast-infected mice had a score of zero, significantly different from that of the uninfected control group. Autonomic functions (Figure 3H), and sensory reflexes (Figure 3I) of infected groups showed scores similar to those of the control group at the end of the study period.

3.2. Recovery and Identification of Fungi Present in the Organs of Infected Immunocompromised BALB/c Mice

Cultures of *A. thermomutatus* UFMGCB 17415 obtained from infected mice lungs were quantified and identified using molecular analysis. Based on the electrophoretic profile of the amplified products, all isolates presented the same band pattern, confirming that the inoculated fungal strain was present in the mice throughout the study period (Supporting Information Figure S1). Similarly, both *R. mucilaginosa* strains were recovered from the brain and spleen of infected mice, showing the same macromorphological pattern (Figure S2).

3.3. Histopathology of Immunocompromised Infected BALB/c Mice

The organs of mice in which the fungi *A. thermomutatus* and *R. mucilaginosa* strains were detected (lungs, spleen, and brain) were subjected to histopathological analysis. Analysis of lungs from BALB/c mice infected with *A. thermomutatus* UFMGCB 17415 demonstrated that the fungus caused an inflammatory process characterized by the presence of a large number of neutrophils around the bronchioles and a moderate number of macrophages, as well as fluid accumulation within the lungs (pulmonary edema). Additionally, there was intense hyperemia (change in blood circulation) with an intense inflammatory infiltrate present primarily around the bronchioles, distributed in a multifocal to coalescent manner. Its composition included a large number of neutrophils (intact and degenerated) and a discrete number of epithelioid macrophages associated with rare structures measuring approximately 3 μm . These had a thin capsule characterized by a clear halo (unstained) surrounding the nucleus, with eosinophilic staining sometimes present in the macrophage cytoplasm (Figure 4). In a small number of bronchioles, a predominantly neutrophilic inflammatory infiltrate was observed. Multifocal and discrete thickening of the alveolar septa was associated with the presence of neutrophils. Additionally, moderate multifocal hyperemia and the presence of amorphous eosinophilic content within the alveoli (edema) were distributed in a multifocal to coalescent manner, along with multifocal areas of discrete hemorrhage. The lung lobes infected with *A. thermomutatus* exhibited changes in all samples analyzed, with the average scores attributed to infection being 6 and 5 points 2 and 5 days after infection, respectively (Figure 5). Mice infected with *R. mucilaginosa* strains did not show significant lesions in the spleen or brain throughout the study (data not shown).

4. DISCUSSION

Various animal models, including BALB/c mice, have been used to evaluate the pathogenicity of opportunistic fungal taxa such as *Aspergillus*^{30–32} and *Rhodotorula*.³³ According to Gonçalves et al.,³² healthy mice did not show symptoms of infection with *A.*

fumigatus; however, 50% mortality was observed in immunosuppressed mice that were inoculated with 10^6 and 10^7 spores, and this increased to 100% when inoculated with 10^8 spores. The analysis of immunocompromised mice in our study showed that strains of both fungi reduced the survival of immunosuppressed infected mice and induced changes in their neuropsychiatric state, which may have been caused by infection (as observed after *R. mucilaginosa* inoculation) or neuroinflammation.

Aspergillus opportunistic infections have gained attention in recent years, primarily because immunocompromised patients are particularly susceptible to infections and experience the highest rates of morbidity and mortality.³⁴ Balajee et al. noted that invasive fungal infections caused by the *A. thermomutatus*/*Neosartorya pseudofischeri* complex are extremely rare; however, this fungus has been reported to cause systemic infections in immunocompromised patients.³⁵ *A. thermomutatus*/*N. pseudofischeri* has been reported to cause pulmonary disease, osteomyelitis, mycotic keratitis, endocarditis, unspecified aspergillosis,¹⁰ and peritonitis in a peritoneal dialysis patient.³⁶ Järvi et al.³⁷ also identified *A. thermomutatus* as an opportunistic agent in a 17-year-old male patient with Hodgkin's disease.^{37,38}

Our results confirm that the Antarctic isolate, *A. thermomutatus* UFMGCB 17415, was capable of inducing an inflammatory process characterized by the presence of neutrophils, macrophages, pulmonary edema, and hyperemia. Similar to the study of Zhang et al., we detected a slightly lower fungal load in the spleen and lungs of animals infected with *A. thermomutatus* compared to the initial inoculum.³⁰ Our findings are also consistent with those of Svirshchevska et al.³⁸ in a study of immunosuppressed BALB/c mice inoculated intranasally with 10^7 conidia of *Aspergillus* spp. They reported signs of lung infection and a 30% mortality rate, similar to that obtained here.

Rhodotorula mucilaginosa is the most widespread species within its genus and a pathogen of emerging concern for immunocompromised patients.³⁹ It is responsible for approximately 70% of infections caused by *Rhodotorula* species.⁴⁰ Our data confirm the presence of fungal load in blood samples of mice infected with *R. mucilaginosa* strains UFMGCB 17448 and 17473. The detection of yeast presence 5 days after infection is concerning, as infections caused by *Rhodotorula* species are generally associated with catheters and can lead to endocarditis and meningitis.⁴¹ Although no tissue changes were observed in the brains of animals infected with *R. mucilaginosa*, its recovery from these brains is notable, indicating that the yeast was able to cross the blood–brain barrier after 72 h of contact, confirming its potential as an opportunistic pathogen in immunocompromised animals. There have been reports of *Rhodotorula*-linked meningitis and brain abscess in immunocompromised hosts.⁴²

5. CONCLUSIONS

Unexplored regions of the planet, including Antarctica, have drawn attention in the context of their resident biodiversity, and particularly to the presence of unknown species and lineages of microorganisms. Antarctic ecosystems have been a focus of studies to understand and characterize extremophilic fungi. Our data confirm that isolates of *A. thermomutatus* and *R. mucilaginosa*, originally obtained from Antarctic permafrost, show *in vivo* virulence against immunocompromised BALB/c mice. Consistent with our previous studies,^{6,21,42} the presence of metabolically viable opportunistically pathogenic fungi in Antarctic permafrost highlights a potentially important impact of climate change in Antarctica, through facilitating the release and further dispersal of fungi and other pathogenic micro-

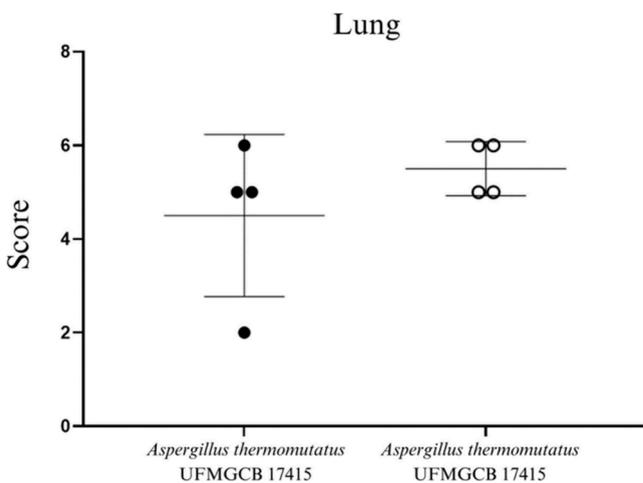


Figure 5. Histopathological evaluation of the lungs of BALB/c mice ($n = 4$) nasally infected and euthanized 2 (black filled circle) and 5 days post-infection (black open circle). The results were checked for normality before being analyzed using the non-parametric Mann–Whitney test.

organisms with potential to affect humans and animals. Surveying and documenting Antarctic microbiota and evaluation of their pathogenic potential are required for understanding, monitoring, and controlling pathogenic microorganisms that may emerge from extreme natural ecosystems.

■ ASSOCIATED CONTENT

Data Availability Statement

The datasets generated during the current study are available from the corresponding authors on reasonable request.

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/envhealth.4c00213>.

Figures S1 and S2 (PDF)

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<https://pubs.acs.org/doi/10.1021/envhealth.4c00213>

Author Contributions

All coauthors meet criteria for authorship. E.C.Q.G., V.N.G., and L.H.R. conceived the study. E.C.Q.G., V.N.G., M.C.d.C.,

D.A.S., S.J., and G.J.C.d.F. performed all *in vitro* and *in vivo* assays. J.B.S.O. and T.A.d.P. performed the histological assays. P.C. interpreted and revised the manuscript. All authors contributed to interpreting the data obtained and drafting the manuscript. All authors read and approved the final manuscript.

Notes

Ethical Conduct of Research: The procedures conducted in this study followed the Brazilian Society of Zootechnics/ Brazilian College of Animal Experimentation guidelines and Federal Law 11794 and were approved by the Ethics Committee on Animal Use of the Universidade Federal de Minas Gerais (CEUA/UFMG, Protocol No. 313/2016).

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study received financial support from Conselho Nacional de Desenvolvimento (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundo Nacional de Desenvolvimento Científico e Tecnológico (FNDCT), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Instituto Nacional de Ciência e Tecnologia (INCT) Criosfera, Programa Antártico Brasileiro (PROANTAR), and Brazilian Navy. P.C. is supported by NERC core funding to the British Antarctic Survey's "Biodiversity Evolution and Adaptation" Team.

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