SCIENTIA PLENA
www.scientiaplena.org.br
VOL. 21, NUM. 09 2025
doi: 10.14808/sci.plena.2025.096201

Inhibitory action of mycocins on airborne fungi isolated from neonatal ICU

Ação inibitória de micocinas sobre fungos anemófilos isolados de UTI neonatal

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(Recebido em 03 de março de 2025; aceito em 01 de agosto de 2025)

Airborne fungi, with the ability to establish themselves and contaminate the air, reduce the life quality of the organisms that circulate there. Invasive fungal nosocomial infections have progressively emerged as a relevant source of morbidity and mortality in patients, especially the immunosuppressed patients. The broad spectrum of antimicrobial activity and the great stability led to the use of *Wickerhamomyces anomalus* as a biocontrol agent, since it could be classified as a low-risk microorganism. The aim of the present study was to evaluate the inhibition of airborne fungi isolated in a Neonatal ICU by mycocins produced by *W. anomalus*. The mycocins containing *W. anomalus* culture supernatant showed β-glucanases activity of 4.2 U/mg. For fungal monitoring and inhibition test in solid environment, the passive sedimentation technique was used. The test consisted of a control plate containing Sabouraud Dextrose Agar, and another composed of culture medium and *W. anomalus* mycocins supernatant. Then, after incubation, the growth and/or inhibition of the collected fungi was observed. The growth of 15 genera was observed on the control plates: *Aureobasidium* spp., *Curvularia* spp., *Emmonsia* spp., *Geotrichun* spp., *Fusarium* spp., *Rhizopus* spp., *Rhodotorula* spp., *Scedosporium* spp., *Chrysosporium* spp., *Trichoderma* spp., *Alternaria* spp., *Aspergillus* spp., *Acremonium* spp., *Penicillium* spp. and *Cladosporium* spp. *Cladosporium* spp. was the most incident in the analyzed period, 40% (86 UFC). Meanwhile, on the test plates there was no growth of microorganisms. As a result, we can see the potential of *W. anomalus* mycocins in the development of new antimicrobial substances.
Keywords: *Wickerhamomyces anomalus*, antimicrobial, intensive care.

Os fungos anemófilos, com a capacidade de se estabelecer e contaminar o ar, reduzem a qualidade de vida dos organismos que circulam no ambiente. As infecções fúngicas nosocomiais invasivas têm surgido progressivamente como uma fonte relevante de morbidade e mortalidade em pacientes, especialmente em imunossuprimidos. O amplo espectro de atividade antimicrobiana e a grande estabilidade levaram ao uso de *Wickerhamomyces anomalus* como agente de biocontrole, uma vez que pode ser classificado como um microrganismo de baixo risco. O objetivo do presente estudo foi avaliar a inibição de fungos anemófilos isolados em uma UTI Neonatal por micocinas produzidas por *W. anomalus*. O sobrenadante da cultura de *W. anomalus* contendo micocina apresentou atividade β-glucanases de 4,2 U/mg. Para o monitoramento fúngico utilizou-se o teste de inibição em meio sólido. O teste consistiu em uma placa controle contendo Ágar Sabouraud Dextrose e outra composta pelo meio de cultura e o sobrenadante da micocina de *W. anomalus*. Após a incubação, foi observada a presença ou inibição dos fungos coletados. O crescimento de 15 gêneros foi identificado nas placas controle: *Aureobasidium spp., Curvularia spp., Emmonsia spp., Geotrichum spp., Fusarium spp., Rhizopus spp., Rhodotorula spp., Scedosporium spp., Chrysosporium spp., Trichoderma spp., Alternaria spp., Aspergillus spp., Acremonium spp., Penicillium spp.* e *Cladosporium spp. Cladosporium spp.* foi o gênero mais incidente no período analisado, representando 40% (86 UFC). Por outro lado, nas placas de teste, não houve crescimento de microrganismos. Como resultado, pode-se observar o potencial das micocinas de *W. anomalus* no desenvolvimento de novas substâncias antimicrobianas.
Palavras-chave: *Wickerhamomyces anomalus*, antimicrobiano, terapia intensiva.

1. INTRODUCTION

Fungi are one of the most abundant, widely distributed, and ubiquitous groups of organisms on Earth [1]. They are capable of uniquely colonizing different substrates and habitats, such as plants, animals, soil, water, and air. These microorganisms can be transported by water, insects, humans, and animals, with the ability to disperse through atmospheric air, known as airborne fungi, enduring large variations in temperature, humidity, pH, and oxygen concentrations [2, 3].

Airborne fungi are considered a fraction of bioaerosols, which may include pollen grains, bacteria, viruses, fungal spores, and their fragments. In this context, the use of bioindicators for environmental monitoring is a valuable tool. They can detect changes in the environment due to the presence of specific pollutants, which in turn affect the biodiversity and particular species of bacteria, fungi, or lichens suspended in the air [4]. They are also sensitive to environmental disorders, showing characteristic external signs according to the pollutant concentration. These fungi, as part of bioaerosols, can also serve as bioindicators of air pollution, plant diseases, and allergic reactions manifested in various clinical forms. They exhibit a wide concentration range in urban areas depending on the interaction between biological and environmental factors [5, 6].

However, airborne fungi can be considered biological pollutants capable of establishing and contaminating indoor air environments, reducing the quality of life for organisms circulating therein [7]. Some of these fungi produce mycotoxins and, upon contact with the human body, can trigger allergic processes, mucosal and skin irritation, fungal infections, and promote exposure of sensitive individuals to their propagules and toxic metabolites [8, 9].

Fungi are underestimated as human disease-causing agents. Complex surgical procedures, loss of integrity of natural barriers, numerous invasive procedures, and prolonged antimicrobial therapy contribute to the worrying increase in these infections, especially in Intensive Care Units [10, 11].

Killer yeasts are those capable of promoting lethal activity against susceptible yeasts. This phenomenon arises from the fulminant action of mycocins — low molecular weight toxic proteins or glycoproteins, considered secondary metabolites secreted by strains that are immune to their own toxin — capable of inhibiting other yeasts without the need for direct cell-to-cell contact [12-14].

These mycocins exhibit great biodiversity in terms of their biochemical, genetic, and mode of action characteristics [15]. The expression of mycocins can be altered depending on variables such as temperature, pH, chemical composition of the medium, and cell concentration. Normally, the optimal activity of mycocins occurs at pH 4.5 at 25 °C, although these values tend to differ to meet the specific conditions of each genus, species, or strains of mycocin-producing yeasts [14-16].

As natural antimicrobials, killer yeasts have been explored for potential applications in food production and preservation, preservatives in formulations and in the biological control of plant, animal, and human pathogens [17, 18].

Wickerhamomyces anomalus was the first mycocin-producing yeast (also called killer yeast) discovered to be capable of inhibiting the growth of both eukaryotic and pathogenic prokaryotic organisms [19, 20].

The ability to inhibit harmful microorganisms in a variety of habitats, broad spectrum of antimicrobial activity, and high stability have led to the use of *Wickerhamomyces anomalus* as a biocontrol agent, as it could be classified as a low-risk microorganism, rarely traced in human samples. Additionally, in vitro activity of *Wickerhamomyces anomalus* against pathogenic yeasts has been proven [21]. Thus, *Wickerhamomyces anomalus* has applicability in processes of the food industry and biocontrol in agricultural and clinical areas [15, 22, 23].

Several mechanisms are proposed to justify the action of mycocins on other microorganisms, including alteration of membrane permeability, inhibition of DNA replication, and inhibition of wall synthesis by β-1,3-glucan synthase, and β-glucan hydrolysis. However, many mechanisms remain unknown and require further study [24]. The yeast in question, *Wickerhamomyces anomalus*, is capable of producing different groups of mycocins with distinct molecular masses. Antimicrobial activity assays confirm the ability of this killer yeast to act on the glucans of the

cell wall [13]. The mechanism of action, not fully elucidated, describes activity on the yeast cell wall by degrading β-glucans through the action of β-1,3-glucanases [21].

Glucan is an important polymer found in bacteria and abundantly in fungal cells. Glucanases-type mycocins act on the hydrolysis of β-1,3-glucan or β-1,6-glucan, a phenomenon that leads to the loss of cytoplasmic components and consequently, cell death. Since mammalian cells lack this constituent in their membrane, the killer mechanism becomes selective for microorganisms. Therefore, this type of mycocins is minimally toxic and with low likelihood of inducing resistance [25-29].

With the rise of resistant pathogens, there is a need for the discovery and development of antimicrobial alternatives. This entails the exploration of minimally aggressive substances, with low toxicity to the human body and optimal safety levels [28, 30].

Considering the potential risk of exposing intensive care patients to opportunistic fungi present in the environment, periodic monitoring is necessary to understand the anemophilic mycobiota in Neonatal Intensive Care Units (NICUs). Furthermore, given microbial resistance to existing products, this study aims to analyze the action of mycocins as an alternative for reducing/controlling airborne fungi in these environments.

2. MATERIAL E METHODS

2.1 Monitoring airborne fungi

A cross-sectional study was carried out from September 2021 to August 2022, in a 10-bed Neonatal Intensive Care Unit (Neonatal ICU). The Hospital in question is located in the municipality of Cascavel, Paraná, offering the region's population high-complexity services in the areas of high-risk pregnancy, traumatology, vascular surgery and neurology, being, in its entirety, the services provided by the Unified Health System – SUS.

The samples were collected through the passive sedimentation technique from the exposure of 9 cm diameter Petri dishes containing Sabouraud Dextrose Agar (SDA) medium for deposition of spores and other fungal structures present in the atmospheric air. The plates were opened for 15 minutes in the morning, always at the same time, at a height of 1 meter from the floor, away from the walls – simulating the position of the cribs and incubators.

After collection, the samples remained incubated at 25°C for fungal growth. 10 days after collection, the Colony Forming Units (CFU) were counted, and each colony was transferred into tubes containing SDA and incubated again for up to 10 days at 25°C for isolation. After this period, the identification of the fungal genera was carried out through the observation of the macroscopic aspects of the colonies, in addition to the microscopic aspect from the microculture between slides and coverslips stained with Lactophenol.

2.2 Inhibition of airborne fungi

2.2.1 Obtaining mycocins from Wickerhamomyces anomalus

The yeast strain utilized was molecularly identified as *Wickerhamomyces anomalus* WA40, and its respective sequence is deposited in GenBank (KT580792 available at https://www.ncbi.nlm.nih.gov/nuccore/KT580792), previously collected from the shores of Lago de Itaipu, located in the state of Paraná, Brazil, and subjected to screening tests to verify mycocins production. It is currently part of the mycological collection at the Laboratory of Clinical Analysis, Teaching, Research, and Extension (LACEPE), stored in three forms: refrigerated, at room temperature, and frozen. Prior to mycocins production, the strains were properly reactivated by inoculation on modified Sabouraud Agar medium (2% agar, 1% peptone, 2% glucose, 1.92% citric acid, and 3.48% dibasic potassium phosphate) at pH 4.7 ± 2°C and incubated at 32°C for 48 hours.

The reactivated strain was inoculated into Roux flasks containing 200 mL of modified Sabouraud broth (1% peptone, 2% glucose, 1.92% citric acid, 3.48% dibasic potassium phosphate) at pH 4.7 and incubated at 25°C for 5 days. Following this period, the broth was centrifuged at 6000 rpm for 10 minutes, yielding the supernatant, which was sterilized by a 0.22 μm membrane filter and stored at 4°C [28].

2.2.2 Determination of β-glucanases activity

The determination of β-glucanases present in the culture supernatant filtrate of *W. anomalus* WA40 containing mycocins was performed according to Miller (1959) [31] with some adaptations, using 1% laminarin (obtained from *Laminaria digitata*) in 50 mM acetate buffer, pH 5.0, and a standard glucose curve. The reaction was prepared using 62.5 μL of the culture supernatant sample of *W. anomalus* WA40 and 125 μL of laminarin. The solution was incubated at 37°C for 10 minutes. After this period, 100 μL of the solution was added to 100 μL of 3,5-dinitrosalicylic acid (DNS) to stop the reaction. The same solution as the test without laminarin was used for the blank. Then, the solutions were incubated in boiling water for 5 minutes with subsequent addition of 500 μL of water. The absorbance was read at 550 nm by a spectrophotometer. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute of reaction, defined as U/min/mL, under the described conditions.

Protein quantification was performed using the method based on the absorption of Coomassie Brilliant Blue G-250 reagent, as proposed by Bradford (1976) [32]. For the preparation of the reaction, 1 mL of the Bradford Reagent was mixed with 100 μL of the supernatant containing mycocins from *W. anomalus*. The mixture was left at room temperature for 5 minutes and then read at 595 nm in a spectrophotometer. The total protein concentration was expressed in mg/mL. The specific activity of β-glucanases was calculated by dividing the concentration of enzymatic activity by the concentration of proteins, and the values were reported in U/mg. The assay was performed in triplicate.

2.2.3 Solid medium inhibition test

In parallel with the monitoring performed by the passive sedimentation technique, inhibition of airborne fungi in solid medium containing mycocins produced by *W. anomalus* was also observed.

Test media were prepared from Sabouraud Dextrose Agar with mycocins supernatant from *W. anomalus* containing 3.4 U/mg of β-glucanases.

The media were poured into Petri dishes and exposed for 15 minutes at a height of 1 meter from the floor in the Neonatal ICU. Subsequently, they were kept at room temperature for 5 to 10 days.

2.2.4 Antimicrobial activity by macrodilution method

For the macrodilution test, the M38-A method from the Clinical and Laboratory Standards Institute (CLSI) [33] for filamentous fungi was used with some adaptations. The fungus tested was the most incident in the Neonatal ICU during the study period – *Cladosporium* spp. The filamentous fungus was cultivated in ASD at room temperature for 7 days. The inoculum was prepared by adding 10 mL of 0.9% NaCl solution with 1 drop of Tween 20. The resulting solution of conidia and hyphal fragments was transferred to a sterile conical bottom tube; a 10-minute wait for particle sedimentation was observed, and the upper homogeneous suspension was adjusted to an optical density of 0.069-0.11 (transmittance of 80-82%) at 625 nm. Standard microbial inocula were adjusted to a concentration of 10^3 CFU/mL, the solutions were homogenized in 10 mL of Mueller Hinton broth (MH) and distributed (500 μL) into tubes.

The supernatant containing mycocins was diluted in sterile distilled water and added to the tubes (500 µL), resulting in the following concentrations of β-glucanases: 0.13, 0.26, 0.52, 1.05, 2.1, and 4.2 U/mg. Readings were taken at 48 hours, such that the last dilution showing inhibition of microbial growth was recorded as the result compared to positive and negative controls. Aliquots of 10 µL were taken from the result tubes and plated on SDA plates to confirm complete inhibition. The test was performed in duplicate.

3. RESULTS AND DISCUSSION

3.1 Monitoring of airborne fungi in the Neonatal ICU

Potentially pathogenic fungi were isolated in the environment during the studied period. Of the 52 collections in the Neonatal ICU environment, 43 showed growth of fungal CFUs. With 213 CFU in total and 15 genera identified – *Aureobasidium* spp., *Curvularia* spp., *Emmonsia* spp., *Geotrichun* spp., *Fusarium* spp., *Rhizopus* spp., *Rhodotorula* spp., *Scedosporium* spp., *Chrysosporium* spp., *Trichoderma* spp., *Alternaria* spp., *Aspergillus* spp., *Acremonium* spp., *Penicillium* spp. and *Cladosporium* spp. Of these, 14 filamentous fungi and 1 yeast genus - *Rhodotorula* spp (Table 1).

The fungal genus with the highest incidence in the period, with 40,4% of the total CFU isolated, corresponded to the genus *Cladosporium* spp. (86 UFC), followed by *Penicillium* spp. with 20,7% (44 CFU). The genera *Geotrichum* spp., *Emmonsia* spp. and *Curvularia* spp. showed 0.5% incidence in isolated CFU, represented by only 1 CFU each, identified only in the spring season.

Table 1. Incidence of fungi in the Neonatal ICU of a hospital in Paraná, Brazil.

Fungal Incidence		
Identified fungal genera	CFU	%
Curvularia spp.	1	0,5
Emmonsia spp.	1	0,5
Geotrichum spp.	1	0,5
Scedosporium spp.	2	0,9
Aureobasidium spp.	3	1,4
Fusarium spp.	3	1,4
Trichoderma spp.	3	1,4
Rhodotorula spp.	6	2,8
Rizhopus spp.	6	2,8
Alternaria spp.	14	6,6
Aspergillus spp.	14	6,6
Chrysosporium spp.	14	6,6
Acremonium spp.	15	7,0
Penicillium spp.	44	20,7
Cladosporium spp.	86	40,4

The season with the highest amount of isolated CFU was autumn (73 CFU), with a predominance of *Penicillium* spp. (42% - 31 CFU). Summer was the season with the lowest number of identified genera: *Alternaria* spp., *Acremonium* spp., *Aspergillus* spp., *Chrysosporium* spp., and *Cladosporium* spp., with *Cladosporium* spp. the most incident, 20 UFC (65%). In the spring, 12 different genera were identified, the season with the greatest fungal variety, *Cladosporium* spp being the most incident, followed by *Penicillium* spp., 9 CFU (18%) (Table 2).

Table 2. Distribution of fungal incidence by season in the Neonatal ICU of a hospital in Paraná, Brazil.

Distribution Incidence by Season of the Year								
Identified fungal genera	Winter		Autumn		Spring		Summer	
	CFU	%	CFU	%	CFU	%	CFU	%
Acremonium spp.	5	8	7	10	3	6	-	-
Alternaria spp.	7	12	1	1	4	8	2	6
Aspergillus spp.	-	-	9	12	-	-	5	16
Aureobasidium spp.	2	3	-	-	1	2	-	-
Chrysosporium spp.	10	17	2	3	2	4	-	-
Cladosporium spp.	30	51	15	21	21	42	20	65
Curvularia spp.	-	-	-	--	1	2	-	-
Emmonsia spp.	-	-	-	-	1	2	-	-
Fusarium spp.	-	-	1	1	2	4	-	-
Geotrichum spp.	-	-	-	-	1	2	-	-
Penicillium spp.	-	-	31	42	9	18	4	13
Rhodotorula spp.	-	-	6	8	-	-	-	-
Rizhopus spp.	5	8	1	1	-	-	-	-
Scedosporium spp.	-	-	-	-	2	4	-	-
Trichoderma spp.	-	-	-	-	3	6	-	-
Total	59	100	73	100	50	100	31	100

The hospital environment is undoubtedly a source of fungal microorganisms capable of promoting allergic reactions and sensitization of atopic individuals, as well as possible fungal infections of the most diverse etiologies, corroborating the studies of anemophilic mycobiota made by different authors [3, 8, 9, 34, 35].

Fifteen different fungal genera were isolated in the Neonatal ICU environment from this study, predominantly *Cladosporium* spp. and *Penicillium* spp. Airborne fungal contamination in Neonatal ICUs has been a topic rarely addressed in studies in Brazil, but it is of great importance due to the appearance of fungal agents in nosocomial infections [36, 37].

Borba et al. verified the presence of airborne fungi and incubator contaminants in a Neonatal ICU and found *Aspergillus* spp., *Cladosporium* spp., *Candida* spp. and *Nigrospora* spp. [38]. With regard to the nosocomial environment, Molina et al. and Lobato et al., when studying hospital environments and fungal dispersion, were able to perceive that events of disinfection, ventilation and personnel transit are determining factors for the presence of anemophilous fungal, and in their studies they verified the prevalence of the genus *Cladosporium* spp. [8, 39].

Cladosporium spp. dematiaceous fungus, which contains melanin in its cell walls, which gives them a dark color in their spores and hyphae, most often associated with the virulence of the microorganism [40]. Although the higher prevalence of *Cladosporium* spp. is verified in atmospheric air, its isolation in indoor air environments was also verified, as well as the occurrence as one of the most prevalent in hospital environments [3, 41, 42]. Although *Cladosporium* species are rare as human pathogens, they are involved in skin infections, phaeohyphomycosis, and lung infections [7], some of them showing significant worsening, which can represent a huge risk for hospitalized patients. Additional studies also observed the presence of *Cladosporium* spp. as one of the main air pollutants in hospital environments [8, 36].

Because of the relationship that aerobiological processes maintain with the atmosphere, meteorological factors significantly influence the behavior of aerial biological particles [43]. Unlike what was described by Menezes et al. (2004) [35] where the summer season had the highest number of isolated genera. This difference is related to the climatic aspects of each region, as well as the methodology used for the isolation of microorganisms in each study [44].

3.2 Inhibition of airborne fungi by mycocins from *Wickerhamomyces anomalus*

3.2.1 Determination of β-glucanases activity

The cell wall of microorganisms is composed of polysaccharides, such as β-glucans. β-glucanases can activate a cell lysis system and act by hydrolyzing and degrading glucans, including β-1,3;1,6-glucans, present in the cell wall of some microorganisms [45, 46].

In this study, the specific activity of β-glucanases obtained from the culture of *W. anomalus* WA40 was 4.2 U/mg. Rosseto et al. (2022) [46] obtained, from the same study, a quantity of 2.47 U/mg. Calazans et al. (2021) [47] reported 0.40 U/mg of specific activity in mycocins produced by *W. anomalus* [47]. Discrepancies in β-glucanases production may be related to the strain and cultivation optimization, as well as cultivation conditions, pH, temperature, inoculum density, and incubation time.

3.2.2 Antimicrobial activity in solid medium

The solid medium test was conducted to evaluate the antifungal activity of mycocins produced by *W. anomalus*. The control plate and the test plate were subjected to equal exposure times. In Figure 1, growth of the fungi sedimented therein can be observed on the control plate (side A); on the test plate (side B), the supernatant containing mycocins from WA40 (concentration of 3.4 U/mg of β-glucanases) was added to the culture medium, thereby verifying total inhibition of airborne fungi.

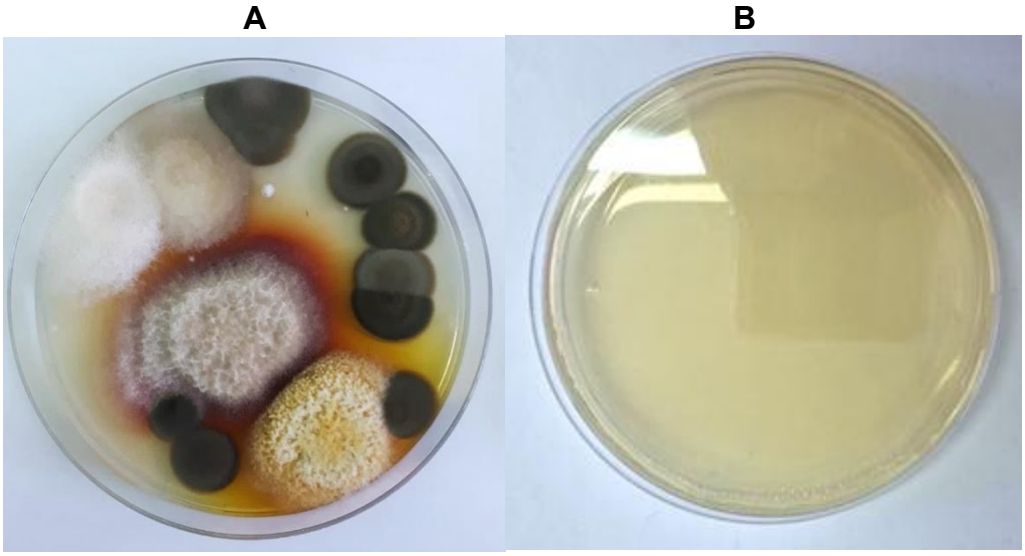

Figure 1. Antimicrobial activity of mycocins in solid medium. (A) Sabouraud Agar culture medium. (B) Culture medium supplemented with 3.4 U/mg of β-glucanases obtained from Wickerhamomyces anomalus WA40 showing no fungal growth.

Performing the antimicrobial activity test on solid medium is a highly effective method as it provides a visual reading of the action of mycocins on the tested microorganism. This was demonstrated in the work of Junges et al. (2020) [48], who found total inhibition of multidrug-resistant strains of *Acinetobacter baumannii* isolated from human biological samples, in the study by Silva et al. (2024) [49], the inhibition of *Malassezia pachydermatis* isolated from the ear canal of dogs was demonstrated, and in the study by Calazans et al. (2021) [47] where inhibition of strains of *Staphylococcus aureus* isolated from meats could be observed. Both results occurred due to the presence of mycocins from *W. anomalus* in the culture medium. In this study, the tested

strains exhibited growth inhibition when inoculated into the culture medium containing 3.4 U/mg of β-glucanases.

3.2.3 Antimicrobial activity by broth macrodilution method

The macrodilution test was conducted to determine the minimum inhibitory concentration of β-glucanases present in the supernatant of *W. anomalus* WA40 capable of preventing the growth of C*ladosporium* spp. strains. Figure 2 demonstrates inhibition when using a concentration of 4.2 U/mg of β-glucanases. Turbidity of the broth was observed for reading, and the last dilution showing inhibition of microbial growth was taken as the result.

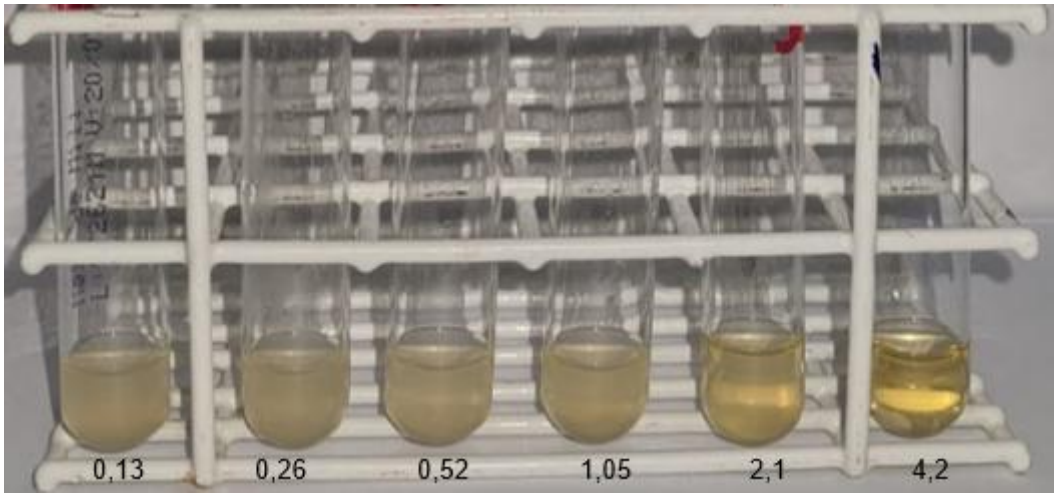

Figure 2. Reading of the macrodilution test showing fungal turbidity at β-glucanases concentrations (U/mg) where growth of Cladosporium spp. strains occurred.

The results showed that mycocins present in the supernatant of WA40 at a concentration of 4.2 U/mg of β-glucanases were able to inhibit 100% (40/40) of the tested *Cladosporium* spp. strains. When using the concentration of 2.1 U/mg, 35% (14/40) of the strains showed sensitivity, whereas at the concentration of 1.05 U/mg, only 5% (2/40) of the strains were susceptible to the supernatant of *W. anomalus* WA40. No inhibition of the strains was observed at other concentrations.

Menezes et al. (2022) [50] considered the broth dilution test as low-cost and easy to perform, as they tested the susceptibility of *Cladosporium sphaerospermum* Penz to Citral. Paris et al. (2016) [28] reported that mycocins showed antimicrobial activity through broth microdilution test when tested against *Candida albicans* yeasts. In this study, through the broth macrodilution test, it was verified that *Cladosporium* spp. strains were inhibited by β-glucanases present in the supernatant of *W. anomalus* cultivation.

4. CONCLUSION

The results of this study clearly showed that hospital environments, especially the Neonatal Intensive Care Unit, undoubtedly constitute sources of potentially pathogenic fungal microorganisms throughout the four seasons of the year. Furthermore, we concluded that mycocins produced by *W. anomalus* (WA40) had inhibitory action on all airborne fungi isolated from the Neonatal ICU in solid medium, which may be attributed to the action of β-glucanases. Thus, we can affirm that these mycocins have pharmacological antimicrobial potential and could potentially be used in formulations for indoor air quality control, especially in patient care settings.

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