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Original Article

Toxigenic fungus isolated from oysters (*Crassostrea* spp.) farmed in the macrotidal region of the Amazonian coast

Fungo toxigênico isolado de ostras (*Crassostrea* spp.) cultivadas na região de macromarés da costa amazônica

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Abstract

Identifying filamentous fungi associated with commercially important and farmed mollusks is essential to ensure food safety and successful production. Therefore, this study aimed to investigate toxigenic filamentous fungi isolated from oysters (*Crassostrea* spp.) collected from an oyster farm in North-eastern Brazil. Twenty oyster samples belonging to the genus *Crassostrea* spp. were collected during the rainy season of 2023 for fungal isolation and subsequent molecular analysis. Fungal isolation followed the methodology recommended by the American Public Health Association, with adaptations. Total DNA extraction was performed using the ITS1 and ITS4 primers. Morphological analysis identified the fungal species *Aspergillus fumigatus* in the farming environment during the rainy season. Inter- and intraspecific distance means were calculated, and a Neighbor-Joining phylogenetic tree was constructed. BLAST analysis revealed high similarity values (99.83%) for *A. fumigatus* specimens, supporting the morphological identification. Both morphological and molecular analyses confirmed the presence of *A. fumigatus* in the analyzed samples. This constitutes the first scientific record of this species in oysters of the genus *Crassostrea* in Brazil. Investigating the potential contamination of oysters by *A. fumigatus* is crucial to safeguarding food safety and public health, as this filamentous fungus is widely distributed in the environment, produces mycotoxins, and causes infections that can lead to cellular damage in humans, among other adverse health effects.

Keywords: *Aspergillus fumigatus*, filamentous fungi, mycotoxin.

Resumo

É necessário identificar fungos filamentosos associados a moluscos comercialmente importantes e cultivados para garantir a segurança dos alimentos e uma produção bem sucedida. Assim, o objetivo do presente estudo é investigar fungos filamentosos toxigênicos isolados de ostras (*Crassostrea* spp.) coletadas em um viveiro de ostras no Nordeste do Brasil. Vinte (20) amostras de ostras pertencentes ao gênero *Crassostrea* spp. foram coletados no período chuvoso de 2023 para isolamento fúngico e posterior análise molecular. O isolamento dos fungos foi realizado de acordo com a metodologia recomendada pela American Public Health Association com adaptações. A extração de DNA total foi realizada utilizandos os primers ITS1 e ITS4. A partir da análise morfológica foi identificado a espécie fúngica *A. fumigatus* em ambiente de cultivo durante o período chuvoso. Foram geradas médias de distâncias interespecíficas e intraespecífica e árvore filogenética de Neighbor Joining. Na plataforma BLAST foi revelado altos valores de similaridade para espécimes de *A. fumigatus* com 99,83% sustentando a identificação morfológica. A identificação morfológica e molecular evidenciou a espécie *Aspergillus fumigatus* nas amostras analisadas. Foi o primeiro registro científico desta espécie em ostras pertencentes ao gênero *Crassostrea* no Brasil. É fundamental investigar a potencial contaminação das ostras por *A. fumigatus* para garantir a segurança dos alimentos e a saúde pública, uma vez que esse fungo filamentoso, amplamente distribuído no meio ambiente, produz micotoxinas e causa infecções que podem levar a danos celulares em humanos, entre outros efeitos negativos.

Palavras-chave: *Aspergillus fumigatus*, fungos filamentosos, micotoxinas.

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1. Introduction

Aquaculture - i.e., the process of breeding aquatic animals in controlled environments - plays an increasingly important role in providing animal protein for the global population (Schreiber et al., 2021). Oyster farming, or oyster growing, stands out for its relevant role in food and nutritional security, for generating job positions and income, and for its socioeconomic impact on riverside communities (Rocha et al., 2013).

Bivalve mollusks, such as oysters, scallops and mussels, play key role in global aquaculture. Bivalves have dominated marine coastal aquaculture production worldwide, in 2018, and accounted for more than half of the total bivalve production, with 17.7 million tons (Fao, 2020). This hegemony translates into nutritional benefits for the world's population; the consumption per capita of fish, including bivalves, reached 20.5 kg/person/year in that same year (Fao, 2020). Oysters-intake benefits are balanced against the danger of exposing consumers to polluting substances likely accumulating in mollusks' edible parts, a fact that represents a risk factor for human health (Oliveira, 2012). Oysters are filter-feeding organisms that eat by pumping water into their body. This process makes them susceptible to the accumulate substances and microorganisms found in the environment in their bodies, and it can pose potential risk to human health, since these mollusks are consumed as raw food (Sousa et al., 2023).

The presence of fungi in food has become a growing concern for food safety and public health, particularly because several filamentous species can produce mycotoxins with high toxic and carcinogenic potential. Studies conducted in Brazil have shown that both food-processing environments and food products, such as dairy items, harbor a wide diversity of filamentous fungi, with particular emphasis on the genera *Aspergillus*, *Penicillium*, and *Cladosporium*, which compromise product quality and pose real risks to consumers (Sousa et al., 2023). In this context, natural compounds have received increasing attention as promising alternatives for controlling these fungi. Phenolic extracts obtained from *Capsicum chinense* (biquinho pepper), for example, have demonstrated antifungal activity against food-relevant pathogens such as *Sclerotinia sclerotiorum* and *Rhizopus stolonifer*, highlighting the potential of innovative strategies to mitigate the risks associated with fungal contamination (Santos et al., 2024). Nevertheless, the occurrence of toxigenic fungi in bivalves, particularly oysters, remains poorly documented, underscoring the urgent need for further investigations in this area.

It is necessary to conducting studies focused on investigating toxigenic fungal species living in oysters' internal organs to enable safe mollusks' dietary consumption, to prevent toxic infections in humans after eating shellfish contaminated with fungi, as well as to help develop oyster farming. Genus *Aspergillus* is one of the main filamentous fungi genera accountings for producing mycotoxins, besides triggering different clinical conditions in humans, such as opportunistic infections, allergies and toxicosis (Howard, 2002; Egbuta, et al., 2017). Species belonging to this group comprise saprophytic fungi, which play important role in carbon and nitrogen cycling processes (Park et al., 2017; Van de Veerdonk et al., 2017). The investigated genus comprises approximately 451 species (Steenwyk et al., 2019) that have great relevance in the food and pharmaceutical industries, but that can also have major negative impacts as pathogens in plants and animals, including humans (El-Hawary et al., 2020).

Considering the foregoing, it is essential to identify filamentous fungi associated with commercially important and farmed mollusks to ensure food safety and successful production. Thus, the aim of the present study was to investigate toxigenic filamentous fungi isolated from oysters (*Crassostrea* spp.) collected in an oyster farm in North-eastern Brazil.

2. Materials and Methods

2.1. Study site

The study was carried out in Raposa municipality, which is in the urban-agglomeration micro-region of São Luís Island, Maranhão State, at the following coordinates: 02°25′22" S and 44°05′21" W. The study site covers approximately 64 km^2 and encompasses Ilha das Ostras (Oysters Island), which was selected for research development purposes due to its important role as oyster farming and trading center (see Figure 1).

The investigated region presents tropical climate featured by mean annual temperature close to 26°C. It is in the intertropical convergence zone between equatorial and tropical climate patterns, and presents two distinct seasons: rainy season, from January to June; and dry season, from July to December. Mean annual rainfall rate reaches 2,100 mm. Rainfall events take place over the year and they contribute to the region's rich biodiversity (Inmet, 2007).

2.2. Fungal collection and isolation

Twenty (20) samples of oysters belonging to genus *Crassostrea* spp. were collected in January 2023. They were placed in isothermal boxes filled with water captured from their habitat and transported to the Microbiology, Pathology and Biotechnology Laboratory (MIPABIO - Laboratório de Microbiologia, Patologia e Biotecnologia) - of State University of Maranhão (UEMA - Universidade Estadual do Maranhão). Fungal isolation was carried out based on the methodology recommended by the American Public Health Association (APHA, 2012), with modifications: 25 g of oysters' soft body was collected and homogenized for subsequent separation of 3g of it in three test tubes filled with Potato-dextrose (P.D.) culture medium. It was done to carry out serial dilutions for plating purposes in Petri dishes added with Potato-Dextrose-Agar (P.D.A.). Fungal incubation and growth were carried out in B.O.D. (Biological Oxygen Demand) germination chamber, at 25 ± 2°C and 12-h photoperiod. The obtained strains were deposited in Prof. Gilson Soares da Silva Mycotheque (MGSS), at the Agricultural Sciences Department of the Agricultural Biotechnology Center at UEMA's Phytopathology Laboratory, under registration number MGSS475.

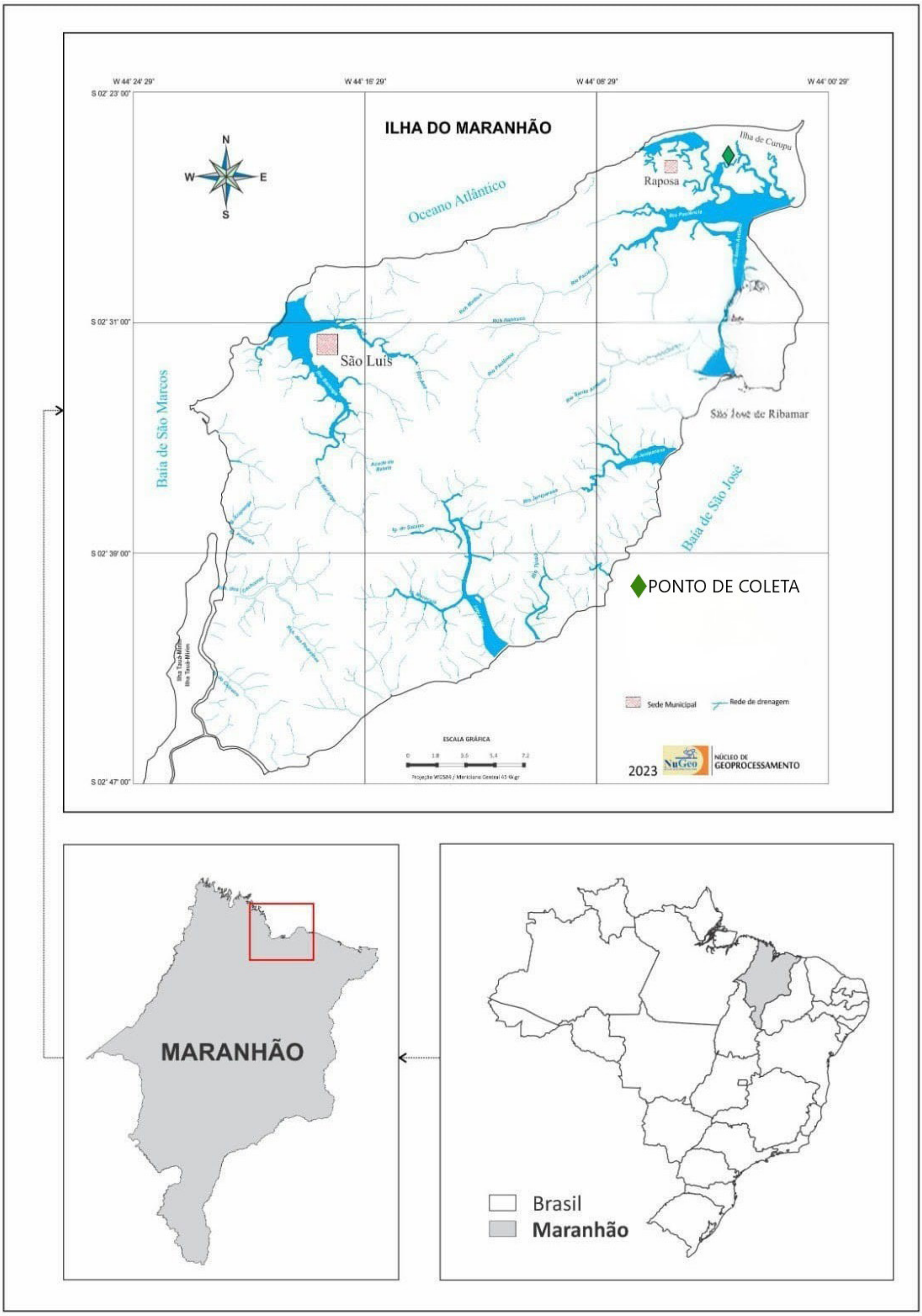

Figure 1. São Luís Island, with emphasis on the study's collection site, and on the collection point in an oyster farming environment in Ilha das Ostras, Raposa municipality (MA). 190x276mm (300 × 300 DPI). Source: Geoprocessing Center of State University of Maranhão (2023).

2.3. Molecular analysis

Total DNA extraction was performed based on the protocol by Kurtzman and Fell (Kurtzman and Fell, 1998), with modification. ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers were used to amplify the isolates' DNA (Gardes and Bruns, 1993; Van de Veerdonk et al., 2017).

Molecular analyses were performed in BioDit 7.0.5.2 (Hall, 1999) software, which was used to edit the 28S gene's ITS (Internal Transcribed Spacer) sequences. These sequences were aligned in CLUSALW software by using default parameters (Thompson et al., 1994). Three (3) Genbank sequences of *Aspergillus fumigatus* and 14 additional sequences of toxigenic species belonging to genus *Aspergillus* were added to the database. The sequence used in the present study was plotted on the BLAST Bioinformatics Platform (Basic Local Alignment Search Tool - Blast.ncbi.nlm.nih.gov/blast.cgi) to investigate its degree of similarity. Tamura Nei (TrN) was selected as the best evolutionary model in jModelTest (Darriba et al., 2012) software, based on the Akaike Information Criterion (AIC). MEGA 11 (Tamura et al., 2021) software was used to set the mean interspecific and intraspecific distance values, and the Neighbor Joining's (NJ) phylogenetic tree. Groupings' significance was estimated through bootstrap analysis (Felsenstein, 1985).

3. Results

Based on morphological analysis, in agreement with the literature, the fungal species *Aspergillus fumigatus* was identified. This species was isolated from oyster samples obtained from the farming environment during the rainy season, with an average concentration of 2.1×10^{-2} CFU/mL.

Martins et al. (2005) and micromorphological analysis allowed identifying *A. fumigatus*, which was first described back in 1864, by Doctor Georg W. Fresenius. This species presents hyaline, smooth-wall conidiophores with balloon-shaped vesicles. Conidial heads are columnar, uniseriate and present one single line of phialides (see Figure 2).

The database set for the ITS region of gene 28S comprised 17 sequences recorded for *A. fumigatus* and toxigenic species belonging to genus *Aspergillus*. They encompassed 565 base pairs (bp) - 411 conserved sites and 148 varying sites. The BLAST platform recorded high similarity values for *A. fumigatus* specimens (99.83%) and it substantiated their morphological identification.

The genetic divergence matrix generated based on the Tamura Nei algorithm did not show variation between the *A. fumigatus* haplotype collected in the study site and the Genbank sequences (see Table 1).

Based on the neighbors-joining phylogenetic tree, the herein analyzed specimens formed a clade, which was substantiated by other Genbank sequences of *A. fumigatus* - 100% bootstrap - (see Figure 3).

4. Discussion

The presence of *Aspergillus fumigatus* in farmed oysters, even at low concentrations, is a relevant finding for public health, as it demonstrates that these bivalves can accumulate spores of opportunistic fungi in their tissues, with the potential to transmit mycotoxins to consumers. It is plausible that the rainy season favored the introduction of these fungal spores into the farming area, an occurrence previously reported in coastal environments by Santos et al. (2017) and Borzykh and Zvereva (2018).

Filamentous fungi belonging to genus *Aspergillus* are known for producing mycotoxins, which are biologically active molecules with low molecular weight. These substances are generated as part of the secondary metabolism of certain filamentous fungi and they can be toxic to vertebrate animals after being metabolized (Maia et al., 2021).

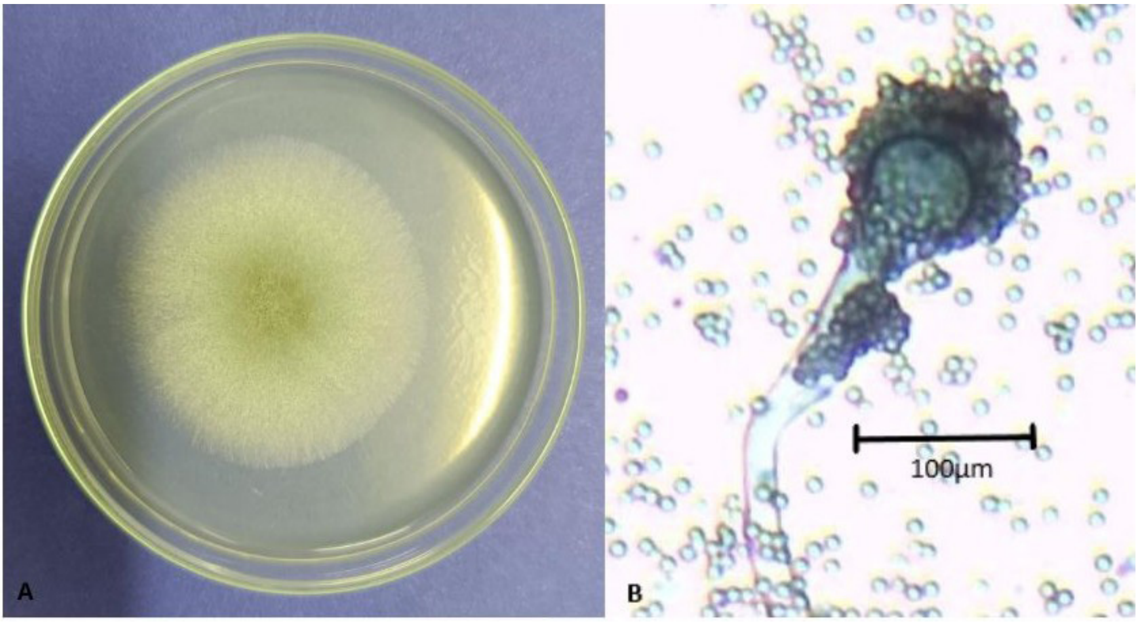

Figure 2. A) Colony of *A. fumigatus* isolated in B.D.A. medium and; B) Methylene blue-stained A. *fumigatus* conidiophore observed under Zeiss optical microscope at 100x magnification.

Table 1. Genetic divergence observed for the 28S gene's ITS region based on using the Tamura Nei algorithm for filamentous fungi belonging to genus *Aspergillus*.

Species/specimens	**Interspecific divergence (%)**																
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
1. *Aspergillus fumigatus* 17E07*	-																
2. *Aspergillus fumigatus* NR_121481	0.0																
3. *Aspergillus fumigatus* MT635279	0.0	0.0															
4. *Aspergillus fumigatus* MT597433	0.0	0.0	0.0														
5. *Aspergillus clavatus* NR_121482	4.0	4.0	4.0	4.0													
6. *Aspergillus carneus* NR_135402	5.4	5.4	5.4	5.4	6.2												
7. *Aspergillus niger* NR_111348	4.8	4.8	4.8	4.8	6.7	4.6											
8. *Aspergillus terreus var_subfloccosus* NR_149331	5.1	5.1	5.1	5.1	6.2	1.7	5.1										
9. *Aspergillus parasiticus var_globosus* NR_151784	6.7	6.7	6.7	6.7	6.9	6.7	6.4	7.2									
10. *Aspergillus flavus* NR_111041	6.1	6.1	6.1	6.1	6.4	6.1	5.9	6.7	0.9								
11. *Aspergillus candidus* NR_077149	4.5	4.5	4.5	4.5	5.1	5.6	6.1	5.9	6.9	6.4							
12. *Aspergillus parasiticus var globosus* NR_151784	6.7	6.7	6.7	6.7	6.9	6.7	6.4	7.2	0.0	0.9	6.9						
13. *Aspergillus ochraceus* NR_077150	7.8	7.8	7.8	7.8	8.1	7.2	8.4	8.4	7.5	6.9	6.9	7.5					
14. *Aspergillus versicolor* NR_131277	11.1	11.1	11.1	11.1	12.3	12	12.7	11.53	12.86	12.86	11.42	12.8	12.5				
15. *Aspergillus ustus* NR_131284	11.7	11.7	11.7	11.7	12.7	12.6	13.3	12.3	13.4	13.1	12.6	13.4	12.3	7.2			
16. *Aspergillus nidulans* NR_133684	11.4	11.47	11.47	11.47	12.65	12.37	12.99	11.8	13.4	13.4	11.7	13.4	13.4	0.9	6.4		
17. *Aspergillus versicolor* NR_131277	11.1	11.1	11.1	11.1	12.3	12.0	12.7	11.5	12.8	12.8	11.4	12.8	12.5	0.0	7.2	0.9	-

*Species of this study

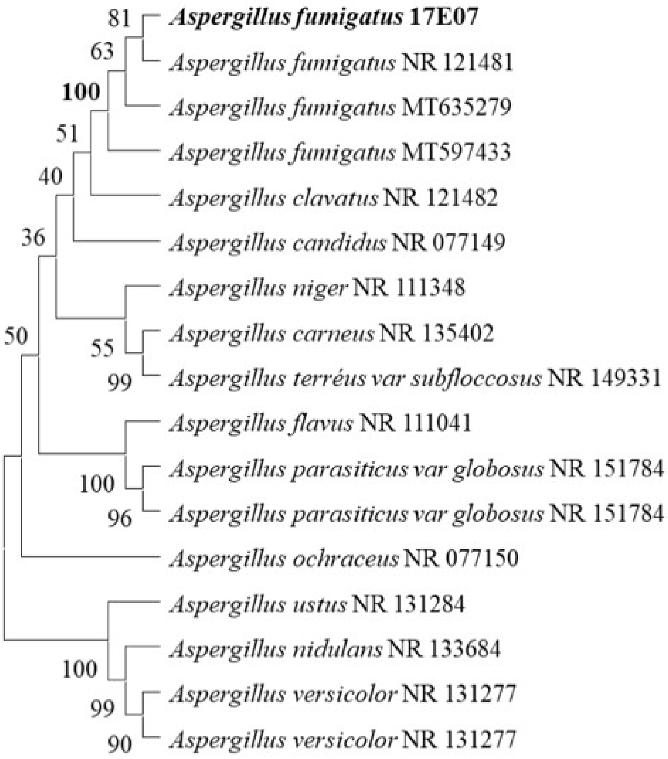

Figure 3. Neighbors-joining phylogenetic tree set for species belonging to genus *Aspergillus*, collected in the present study (in bold) and obtained from Genbank.

Approximately 300 different mycotoxins are produced by filamentous fungi; among them, one finds toxins, such as fumagillin, glyotoxin, verruculogen and viriditoxin, which are produced by *A. fumigatus* (Sweeney and Dobson, 1998). Glycotoxin is a toxic compound that has different harmful effects on the human body. Immunotoxicity stands out among its main effects. In other words, glotoxin can compromise the immune system and make individuals more susceptible to infections and different diseases. Nephrotoxicity, in its turn, can damage individuals' kidneys and lead to renal failure. Hepatotoxicity can damage the liver and impair its vital functions, whereas genotoxicity can damage individuals' DNA, thus increasing the risk of mutations and, consequently, of cancer (Shankar, 2013).

In addition to producing secondary metabolites with toxic and carcinogenic potential, such as gliotoxin, *A. fumigatus* ranks among the 20 fungal species most frequently associated with opportunistic infections, particularly pulmonary infections, in immunocompromised individuals (Kupfahl et al., 2008). This species has also been reported to induce cellular toxicity (Gniadek et al., 2011) and to impair immune system function through gliotoxin synthesis (Pahl et al., 1996).

Mycological and toxicological studies carried out with scallop species Mizuhopecten Yessisis, based on using Immunoenzymatic Assay (ELISA), have evidenced that these mollusks' internal organs accumulate toxins produced by filamentous fungus *A. flavus*. Glyotoxin accumulation in soft tissues of the bivalve mollusk *Mythilus edulis* was investigated in an assay carried out in France, which showed that *A. fumigatus* can pose risks to seafood consumers, since glyotoxin can be secreted in seawater and accumulate in this mussel without any visible effect (Grovel et al., 2002).

Research conducted by Sallenave-Namont et al. (2000) has found toxigenic fungi, including those belonging to genus *Aspergillus*, in mussels, bivalves, sediments and seawater. The previously cited study also assessed the toxicity of these fungi using *Artemia salina* larvae, a crustacean species highly sensitive to mycotoxins. Results have evidenced that fungi deriving from marine environment can produce toxins that pose real intoxication risk to individuals who consume contaminated seafood.

The analysis applied to the structure of a set of filamentous fungi isolated from mussel *Crenomythilus grayanus* and *Modiolus modiolus* has identified, among other species, twelve species belonging to genus *Aspergillus*, namely: *A. candidus*, *A. janus*, *A. mangini*, *A. nidulans*, *A. niger*, *A. ochraceus*, *A. parasiticus*, *A. speluneus*, *A. sulphureus*, *A. versicolor*, *Aspergillus* sp. 1 and *Aspergillus* sp. 2. *A. fumigatus* incidence was not identified in this analysis (Zvereva and Vysotskaya, 2005). A similar study conducted in Japan reported to have isolated genus *Aspergillus* from scallop *Mizuhopecten yessoensis* (Borzykh and Zvereva, 2018).

Santos et al. (2017) have analyzed the microbiota of scallop *Nodipecten nodosus* and reported incidence of filamentous fungi, including the ones belonging to genus *Aspergillus*, in Rio de Janeiro State, Brazil. This outcome led to potential public health concerns. Borzykh and Zvereva (2014) have analyzed the microbiota of oyster *Crassostrea gigas*, of scallop *Mizuhopecten yessoensis* and of mussel *Mythilus trossulus*. They also reported to have isolated fungus *A. fumigatus* from *Mizuhopecten yessoensis* and *Mythilus trossulus*. However, this fungal species was not identified in oyster *Crassostrea Gigas*.

Another study available in the literature also reported to have isolated some filamentous fungi, such as *Aspergillus* spp., from bivalve mollusk *Perna perna* grown in marine farm located in Tarituba Beach, Paraty City, Rio de Janeiro State. The authors of the study highlighted public health concerns regarding the increasing diversity of *Aspergillus* species detected in the organs of the investigated mollusks, as this finding indicates coastal water pollution (Santos et al., 2020). Finally, there are no reports in the literature about oyster genus *Crassostrea* association with fungus *A. fumigatus*. Galimberti This fungal species should also be investigated by taking into consideration the consumption of this oyster, since this fungus produces toxins, such as glyotoxin and mycotoxin, which can be highly harmful to humans, after they are ingested (Egbuta et al., 2017).

From a toxicological perspective, the presence of *A. fumigatus* in organisms intended for human consumption warrants careful attention. Although no chemical analyses were conducted in this study, the possibility of bioaccumulation of these metabolites in edible tissues cannot be excluded, particularly considering that oysters are often consumed raw.

These findings underscore the need to incorporate mycological investigations into sanitary surveillance programs for farmed mollusks. While the National Program for the Hygienic-Sanitary Control of Bivalve Mollusks (PNCMB), the resolutions of the National Council for the Environment (CONAMA), and other international regulations are essential for ensuring hygienic and sanitary quality, their scope remains primarily focused on bacteria, viruses, and algal biotoxins. However, as evidenced in the present study, fungi are also present in mollusks and may pose risks to public health. In this regard, the inclusion of mycological analyses in these monitoring systems would significantly contribute to strengthening food safety.

5. Conclusion

The present study is the first to provide a scientific record of *A. fumigatus* isolated from oysters of the genus *Crassostrea* in both Brazil. Investigating the potential contamination of oysters by *A. fumigatus* is essential to ensuring food safety and protecting public health, as this filamentous fungus is widely distributed in the environment, produces mycotoxins, and can cause infections leading to cellular damage in humans, in addition to other adverse health effects.

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Data Availability Statement

The research data analyzed in this study are not publicly available in any repository or database.

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