

Accessing indoor fungal contamination using conventional and molecular methods in Portuguese poultries

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Abstract Epidemiological studies showed increased prevalence of respiratory symptoms and adverse changes in pulmonary function parameters in poultry workers, corroborating the increased exposure to risk factors, such as fungal load and their metabolites. This study aimed to determine the occupational exposure threat due to fungal contamination caused by the toxigenic isolates belonging to the complex of the species of *Aspergillus flavus* and also isolates from *Aspergillus fumigatus* species

complex. The study was carried out in seven Portuguese poultries, using cultural and molecular methodologies. For conventional/cultural methods, air, surfaces, and litter samples were collected by impaction method using the Millipore Air Sampler. For the molecular analysis, air samples were collected by impinger method using the Coriolis μ air sampler. After DNA extraction, samples were analyzed by real-time PCR using specific primers and probes for toxigenic strains of the *Aspergillus flavus* complex and for detection of isolates from *Aspergillus fumigatus* complex. Through conventional methods, and among the *Aspergillus* genus, different prevalences were detected regarding the presence of *Aspergillus flavus* and *Aspergillus fumigatus* species complexes, namely: 74.5 versus 1.0 % in the air samples, 24.0 versus 16.0 % in the surfaces, 0 versus 32.6 % in new litter, and 9.9 versus 15.9 % in used litter. Through molecular biology, we were able to detect the presence of aflatoxigenic strains in pavilions in which *Aspergillus flavus* did not grow in culture. *Aspergillus fumigatus* was only found in one indoor air sample by conventional methods. Using molecular methodologies, however, *Aspergillus fumigatus* complex was detected in seven indoor samples from three different poultry units. The characterization of fungal contamination caused by *Aspergillus flavus* and *Aspergillus fumigatus* raises the concern of occupational threat not only due to the detected fungal load but also because of the toxigenic potential of these species.

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Introduction

Among agricultural workers, poultry farmers are the ones with higher risks of occupational respiratory diseases (Kogevinas et al. 1999; Senthilselvan et al. 1997; Novak 1994). Exposure to organic dust is one of the most recognized respiratory hazards associated with animal production (Omland 2002).

The term “organic dust” is often used synonymously with “bioaerosol” in the occupational health context and can be defined as “particles with biological origin suspended in air.” Microorganisms such as fungi are small enough to remain airborne once they have been dispersed into the air, and can be found as single organisms and aggregates of cells or spores (Eduard and Halstensen 2009).

Epidemiological studies showed increased prevalence of respiratory symptoms and adverse changes in pulmonary function parameters in poultry workers (Radon et al. 2001; Rylander and Carvalho 2006), corroborating the increased exposure to risk factors, such as fungal load and their metabolites.

Airborne fungi present in poultry facilities include the genera *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp., and less commonly, *Alternaria* sp., *Fusarium* sp., and *Geotrichum* sp. (Sauter et al. 1981; Lee et al. 2006). In Portuguese poultries, specifically, the most prevalent genera in the air are *Rhizopus* sp. (30 %), *Penicillium* sp. (10 %), and also *Aspergillus* sp. (9.7 %), and one of the most frequent species is *Scopulariopsis brevicaulis* (39 %) (Viegas et al. 2012a). The prevalence of *Aspergillus* genus in these settings, considering air and swab samples, was 22.2 %. From the 59 collection points where the *Aspergillus* isolates were obtained, 10 different species were identified. *Aspergillus flavus* was the second (23.7 %) and *Aspergillus fumigatus* the third most frequent (15.3 %) (Sabino et al. 2012).

Isolates from the *Aspergillus flavus* species complex are very important toxigenic fungus that produces aflatoxins which are of great concern because of their health effects on humans and animals (Okoth et al. 2012). Aflatoxins are polyketide-derived compounds produced by *Aspergillus flavus* during growth on crops such as corn, peanuts, cottonseed, and tree nuts (Cary et al. 2012). Aflatoxins comprise a family of toxic secondary metabolites that have been reported to be acutely toxic, teratogenic, and potent carcinogenic and mutagenic

agents. Besides *Aspergillus flavus*, strains from other fungi can also produce these mycotoxins, namely *Aspergillus parasiticus* and *Aspergillus nomius*. These species are frequently found in foodstuffs and animal feeds (Dimitrakakis et al. 2008; Akman et al. 2012).

Aspergillus fumigatus is a common thermophilic fungus found in contaminated animal feed (Scudamore and Livesey 1998; Schneweis et al. 2001; Cavaglieri et al. 2005; Pereyra et al. 2008) and also in human dwellings (Denning 1998). This fungus belongs to the group of microorganisms considered indicators of moisture-damaged buildings (Samson et al. 1994). Its spores are easily spread in the air and therefore pose a high risk of exposure for both animals and humans (Land et al. 1987). This species is a well-known human and animal pathogen causing aspergillosis (Sutton et al. 1996) and other respiratory pathologies (Pena et al. 2010).

As other fungi, isolates belonging to the *Aspergillus fumigatus* complex produce a number of toxins. The most abundant mycotoxin produced by this species is gliotoxin (Orciuolo et al. 2007). The frequency of gliotoxin production in environmental *Aspergillus fumigatus* strains ranged between 0 and 33 % (dos Santos et al. 2003; El-Shanawany et al. 2005; Fischer et al. 1999; Land et al. 1989) and it was detected in 93 % of cultures recovered from patients at a Tertiary Care Cancer Center (Lewis et al. 2005). Gliotoxin is a highly toxic secondary metabolite produced during hyphal growth. This mycotoxin causes damage to the ciliated respiratory epithelium in vitro and this property might assist *Aspergillus fumigatus* in the colonization of the respiratory mucosa (Amitani et al. 1995) and invasion of the respiratory tract (Kyung et al. 2009).

Although viability is crucial in the study of infectious organisms, cultural methods show poor precision and a highly variable underestimation of exposure (Eduard and Halstensen 2009). For this reason, molecular methods have been developed to identify microorganisms independent of cultivability (Viegas et al. 2012b; Eduard and Halstensen 2009).

This study aimed to determine the occupational exposure threat due to fungal contamination caused by *Aspergillus flavus* and *Aspergillus fumigatus* complexes in seven Portuguese poultries using conventional and molecular methodologies, comparing the outputs of both methods.

Materials and methods

Poultry farms

The selected poultry farms were located at Lisbon district, Portugal, and samples were collected between January and May 2011. Selected farms were dedicated to broiler chicken production, where birds are bred to reach slaughter weight as rapidly as possible. Day-old chicks are transferred from hatcheries to the growing farms where they are housed in single-story sheds. The number of chickens in each farm ranges between 11,000 and 74,000, with animal densities ranging between 10 to 100 chickens/m². Farm staffs monitor the condition of the birds daily, adjust feed and water equipment as necessary, and administer vaccines. In some cases, the same workers also catch and transport mature chickens to slaughterhouse.

Sample collection

Air fungal contamination was studied by conventional and molecular methods. Twenty seven air samples of 25 L were collected through impaction method using Millipore Air Tester at 1-m height with a flow rate of 140 L/min, onto malt extract agar (MEA) supplemented with the antibiotic chloramphenicol (0.05 %). Air sampling was performed in the pavilion's interior and outer premises, since this was regarded as the reference site.

Besides impact method, and in order to perform molecular analysis, 18 air samples of 300 L were collected in five of the seven poultries through impinger method using the Coriolis μ air sampler (Bertin Technologies), at 300 L/min airflow rate. Each air sample was collected into a conic sterile tube containing 10-mL sterile phosphate-buffered saline and 0.05 % Triton X-100.

Surface samples were collected by swabbing the surfaces of the same indoor sites, using cotton swabs pre-moistened in a saline solution and a 10×10-cm² stencil disinfected with 70 % alcohol solution between samples according to the International Standard ISO 18593 (2004). The obtained swabs were then plated onto MEA.

Besides air and swab samples, 7 new and 14 aged litter samples were collected from seven poultries in sterilized bags. Each litter sample (10 g—not oven-dried prior to processing, retaining thus its natural water

content), was diluted in 100 mL of sterilized distilled water, agitated for 30 min at 100 rpm and 0.2 mL of this suspension was spread onto triplicate Petri dishes containing malt extract agar (2 %) with chloramphenicol (0.05 g/L) (Table 1).

Preparation and analyses of the collected samples

Conventional methodologies

All the collected samples were incubated at 27 °C for 5 to 7 days. After laboratory processing and incubation, quantitative (colony-forming units—CFU/m³, CFU/m², and CFU/g) and qualitative results were obtained, with identification of the isolated fungal species. For species identification, microscopic mounts were performed using tease mount or Scotch tape mount and lactophenol cotton blue mount procedures. Conventional methodologies were based in culture and included macro and micromorphological identification of the isolates through their characteristics as noted by Hoog et al. (2000).

Molecular methodologies

Five milliliters from the collection liquid were centrifuged at 2,500×g for 10 min and supernatant was removed to leave a 250- μ L pellet that was subsequently used for DNA extraction. DNA was then extracted using the ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research) according to the manufacturer's recommendations.

Confirmation of fungal DNA extraction was obtained by amplifying a fragment of the 18S rDNA gene with universal fungal primers (Wu et al. 2002). Molecular detection of *Aspergillus flavus* and *Aspergillus fumigatus* was achieved by real-time polymerase chain

Table 1 Samples collected for conventional methods and molecular biology analysis

Fungal assessment	Indoor air samples	Outdoor air samples	Surfaces samples	Litter samples	
				New	Aged
Conventional methods	20	7	20	7	14
Molecular biology	12	6	NA	NA	NA

NA not applicable

Table 2 Specific primers and TaqMan probes used in real-time PCR for DNA amplification of isolates belonging to *Aspergillus flavus* and *Aspergillus fumigatus* complex

	Sequence	Reaction conditions	
		Concentration (μM)	Ann T (°C)
<i>A. flavus</i> (Mayer et al. 2003)	F: GTCCAAGCAACAGGCCAAGT	0.5	53
	R: TCGTGCATGTTGGTGATGGT	0.5	
	P: FAM-TGTCTTGATCGGCGCCCG-TAMRA	0.375	
<i>A. fumigatus</i> (Cruz-Perez et al. 2001)	F: CGCGTCCGGTCCTCG	0.375	52
	R: TTAGAAAAATAAAGTTGGGTGTCGG	0.375	
	P: FAM-TGTCACCTGCTCTGTAGGCCCG-TAMRA	0.375	

reaction (PCR) using the iQ real-time PCR detection system (Bio-Rad) (Table 2). The PCR reactions are targeted to *Aspergillus flavus* toxigenic strains only, by using specific primers for the NOR-1 gene, which encodes a protein acting in the aflatoxin biosynthetic pathway (Mayer et al. 2003). This gene is deleted in non-toxicogenic strains of *Aspergillus flavus*. In what concerns *Aspergillus fumigatus*, the primers are specific to the 18S rDNA gene, which is homologous within all the strains from the complex (Cruz-Perez et al. 2001). Reactions included 1× iQ Supermix (Bio-Rad), 0.5 μM of each primer, and 0.375 μM of TaqMan probe in a total volume of 20 μl. Amplification followed a three-step PCR: 40 cycles with denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. The specificity of the primers and probe set was confirmed by testing these primers in DNA extracted from pure cultures of different species from the same genus (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, *Aspergillus versicolor*, and *Aspergillus ochraceus*) and 14 other species corresponding to different fungal genera (data not shown).

Results

Table 3 summarizes the global results concerning the identification and quantification of fungi obtained when conventional methodologies were applied to air, surfaces, and litter samples. *S. brevicaulis* (40.5 %) was the most commonly isolated species in poultries' air and *Rhizopus* sp. (30.0 %) was the most commonly isolated genus. *Penicillium* genus was the third most frequently found (10.1 %) and the most frequently detected in new and used litter from the same setting.

Among the *Aspergillus* genus, *Aspergillus flavus* and *Aspergillus fumigatus* presented different prevalences, namely: 74.5 versus 1.0 % in the air samples, 24.0 versus 16.0 % in the surfaces, 0 versus 32.6 % in new litter, and 9.9 versus 15.9 % in used litter (Fig. 1).

Molecular methods were applied to identify the presence of aflatoxigenic strains from *Aspergillus flavus* complex and also the presence of strains of *Aspergillus fumigatus* complex in 18 air samples belonging to five poultry units (12 indoor samples from six different pavilions and the corresponding six outdoor reference samples).

Table 3 Most frequent fungi identified in poultries through conventional methods

Air ^{*a}	Freq. (%)	Surfaces	Freq. (%)	New litter ^{*b}	Freq. (%)	Used litter ^{*b}	Freq. (%)
<i>S. brevicaulis</i>	40.5	<i>A. versicolor</i>	31.4	<i>Penicillium</i> sp.	59.9	<i>Penicillium</i> sp.	42.3
<i>Rhizopus</i> sp.	30.0	<i>A. flavus</i>	16.5	<i>Alternaria</i> sp.	17.8	<i>Scopulariopsis</i> sp.	38.3
<i>Penicillium</i> sp.	10.1	<i>F. solani</i>	15.3	<i>Cladosporium</i> sp.	7.1	<i>Trichosporon</i> sp.	8.8
<i>Aspergillus</i> sp.	9.7	<i>Penicillium</i> sp.	8.8	<i>Aspergillus</i> sp.	5.7	<i>Aspergillus</i> sp.	5.5
Others	9.7	Others	28.0	Others	9.5	Others	5.1

^{*a}Adopted from Viegas et al. 2012a, b, c

^{*b}Adopted from Viegas et al. 2012a, b, c

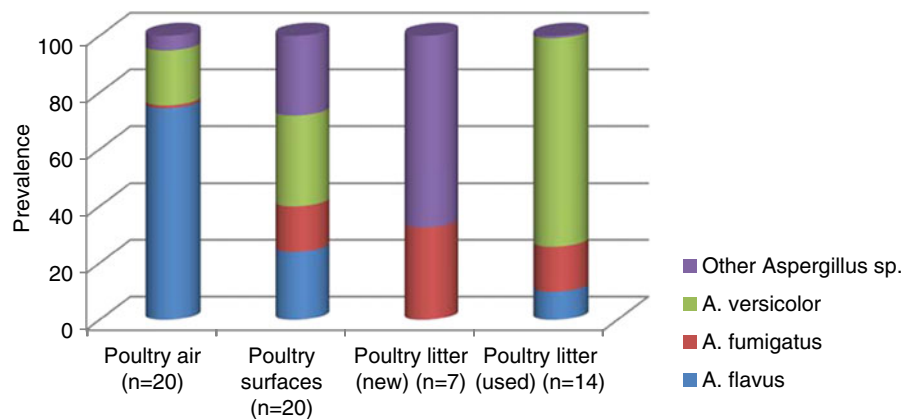


Fig. 1 *Aspergillus* spp. distribution in the collected samples

Aspergillus flavus was identified in five indoor air samples from two different units through conventional methods. However, by real-time PCR, positive results were obtained in three indoor samples from two units (only one unit is coincident with the ones with positive cultural results for *Aspergillus flavus*). The presence of aflatoxigenic strains was detected only in one of the outdoor air samples, also indicating the possible existence of an *Aspergillus flavus* source inside the building

in, at least, one of the cases. Aflatoxigenic strains were not identified (by real-time PCR) in all samples from where *Aspergillus flavus* colonies were detected by conventional methods. On the other hand, we were able to detect the presence of aflatoxigenic strains in pavilions in which *Aspergillus flavus* did not grow in culture (Table 4).

Through conventional methods, *Aspergillus fumigatus* was only found in one indoor air sample. Using real-time

Table 4 Distribution of *A. flavus* and *A. fumigatus* species complex in the collected samples

	Farms	Conventional methodologies (impact method)				Molecular biology (impinger method)	
		Air* ^b (CFU/m ³)	Surfaces* ^b (CFU/m ²)	New litter* ^b (CFU/g)	Aged litter* ^b (CFU/g)		Samples number
<i>A. flavus</i> species complex* ^a	1	5,800	0.3	–	12,500	Identified in: - 3/6 Indoor air samples - 3/4 Surfaces samples - 1/4 Aged litter samples	Detected in 2/4 indoor air samples
	2	–	–	–	–	–	Detected in 2/3 indoor air samples
	3	40	–	–	–	Identified in - 1/1 Indoor air samples	Not detected
<i>A. fumigatus</i> species complex	1	–	–	5,000	–	Identified in 1/2 new litter samples	Detected in 2/4 indoor air samples
	2	80	0.0001	–	10,000	Identified in: - 1/3 Indoor air samples - 1/3 Surfaces samples - 1/3 Aged litter samples	Detected in 3/3 indoor air samples
	3	–	–	10,000	–	Identified in 1/1 new litter samples	Detected in 2/2 indoor air samples

^{*a}Adopted from Viegas et al. 2012a, b, c

^{*b}Total of colonies

PCR for *Aspergillus fumigatus* species complex detection, on the other hand, it was identified in seven indoor samples from three different poultry units. The presence of *Aspergillus fumigatus* species complex was confirmed in two outdoor air samples indicating also the possible existence of an *Aspergillus fumigatus* source inside the building in, at least, one case (Table 4). Both assessed species were found in the farms with less (11,000) and higher number of chickens (74,000) and no correlation with animal density was found.

Discussion

In recent decades, farmers in Europe and North America have enlarged livestock production techniques by using more enclosed and densely stocked housing (confinement buildings). The design of these buildings are similar in which animals, their wastes, and feed, are highly concentrated, resulting in high levels of dusts, gases, microbes, microbial metabolites, and other potential health hazards in the air (Donham et al. 1989).

With more animals, materials, equipments, and workers in the production facility, there is a concurrent increase in three distinct yet interrelated types of airborne materials: (1) bioaerosols, (2) dust (mineral particulates serving as carriers), and (3) odorous volatile compounds (Millner 2009). Bioaerosols are comprised of airborne bacteria, fungi, viruses, and their by-products, endotoxin, and mycotoxin (Oppliger et al. 2008). Regarding fungi, one of the genera more frequently found in poultry farms is *Aspergillus* (Sauter et al. 1981; Lee et al. 2006; Viegas et al. 2012a; Sabino et al. 2012), being *Aspergillus flavus* and *Aspergillus fumigatus* as the most frequently isolated species (Sabino et al. 2012).

Aspergillus fumigatus conidia are extremely well suited for air dispersal since their small size and hydrophobicity allow them to remain airborne for very long periods (O’Gorman 2011). When they eventually settle down, they contaminate all surfaces in contact with the air, and can easily become airborne again creating mini bursts of spores (Rahme 1991). However, *Aspergillus fumigatus* was only found through conventional methods in one air sample, and in less prevalence on surfaces than *Aspergillus flavus*. This may be due to the fact that the ideal temperature for the detection of thermophilic species as *Aspergillus fumigatus* is 40 °C (Fullerenger et al. 2006) since other saprophytic fungal species will not grow at this temperature. Since all the collected samples

were incubated at 27 °C, data about the presence of this fungus in these settings was under estimated with conventional methodologies. Moreover, differences in fungi growth rates may also result in data underestimation, since the fungal species with higher growth rates may inhibit others species’ growth. Finally, underestimated data can result from non-viable fungal particles that may have been collected or fungal species that do not grow in the used culture media, although these species may have clinical relevance in this context (Bartlett et al. 2004; Strachan et al. 1990) as is the case of *Aspergillus fumigatus*.

Due to the easiness of fungal detection, fungi are often used as an indirect indicator of mycotoxins presence in agricultural and occupational settings (Thrane et al. 2004). Thus, besides the fungal load in a specific setting, we must also consider the possibility of mycotoxin presence. Regarding aflatoxin potential exposure, we previously reported that 18 (58.6 %) poultry workers from the same farms displayed detectable levels of AFB1, in contrast to all individuals used as controls who showed no detectable values (Viegas et al. 2012a, b, c). These findings corroborate the hypothesis that occupational exposure to AFB1 by inhalation occurs in poultry production. The same exposure assessments should be performed for gliotoxin, since in a study developed in 2005, this mycotoxin was detected in 93 % of *Aspergillus fumigatus* cultures recovered from patients at a Tertiary Care Cancer Center (Lewis et al. 2005). Biomarker data are a more accurate reflection of exposure assessment because they allow the real measure of AFB1, and other mycotoxin present in the organism, enabling a focus on body burden or total absorbed dose (Watson and Mutti 2004).

Conventional methods for detecting and identifying fungal species are based on isolation, cultivation, and subsequent observation of morphological traits and/or cultural characteristics of colonies, such as their color and texture, conidial size and texture, and conidiophore structure. These methods are time consuming and laborious: days or even weeks may be required to isolate the fungi in culture. Intra- and inter-specific variations in morphological characteristics, due to differences in growth and culture conditions, can also pose serious problems in identification. In addition, not all fungal species can be cultivated on any given media (Wu et al. 2002) and at the same temperature (Zorman and Jersek 2008). Therefore, the cultural analyses may not accurately reflect the true fungal community in a given

sample (Wu et al. 2002; Zorman and Jersek 2008). PCR-based techniques have been widely used in specific and direct detection of DNAs and RNAs of microorganisms from clinical and environmental samples to determine, accurately and quantitatively, the composition of microbial communities. By using PCR primers designed to amplify DNA sequences of a specific group or species of microorganisms, the presence of the microbes can be detected from as little as one or a few target cells (Wu et al. 2002). In this study, real-time PCR using TaqMan probes were used because it is much more specific and sensitive and less contamination-prone than traditional PCR (Jensen and Arendrup 2012). However, no quantitative determinations were performed yet.

Incubation temperature and growth inhibition caused by other species, may justify the increased capacity of detection observed when using real-time PCR. Furthermore, the possibility to detect non-viable spores and also the specificity of the method to detect toxigenic strains (as was the case of *Aspergillus flavus* strains) contribute to the referred increased ability. These situations justify the differences between the obtained results from conventional and molecular methods.

Other studies that applied conventional methods and molecular biology to assess fungal contamination in highly contaminated environments, also revealed different results from both methodologies in what concerns *Aspergillus flavus* (Viegas et al. 2012c), *Aspergillus fumigatus* (Bellanger et al. 2010), and *Stachybotrys chartarum* (Malta-Vacas et al. 2012), being the species/strains more frequently found through molecular biology.

Non-culturable and/or non-viable spores may still be allergenic and cause health problems (Wu et al. 2002). However, in the case of a possible fungal occupational exposure through inhalation, conventional methods offer the advantage of enabling identification and quantification only of viable microorganisms and, therefore, the ones that probably represent higher health risks when comparing with non-viable organisms (Samson et al. 2000; Eduard and Halstensen 2009). Nevertheless, available genetic information is still scarce and scattered for many species (Homer 2003), essentially regarding species complexes. In addition, costs associated with molecular biology techniques are still high, which reinforce the use of the conventional methodologies. Therefore, and in parallel with molecular biology, the use of

conventional methods is still recommended for fungal species confirmation (Borman 2009). Thus, both methods have advantages and limitations when applied to characterize occupational exposure to biological agents in different settings. However, when applied together and through technical intervention of biologists and environmental health technicians, they become complementary tools useful in the evaluation of the setting (Viegas et al. 2012b). This situation was considered to design this study and should be accessed in further studies.

Real-Time PCR was applied only in air samples in our study, but with the obtained data, we can also suppose that the prevalence of isolates belonging to *Aspergillus flavus* complex and *Aspergillus fumigatus* complex in surfaces as well as in the new and aged litter, obtained through conventional methods, should be higher than what was detected. Although we can conclude that there is a potential exposure to gliotoxin due to presence of toxigenic strains (Thrane et al. 2004) considering the *Aspergillus fumigatus* load, further studies about the characterization of the detected *Aspergillus fumigatus* strains gliotoxin producers, are needed.

Furthermore, in this occupational setting, we must consider a potential exposure to more than one mycotoxin, since more than one fungal species recognized as mycotoxin producers were found (Viegas et al. 2012a, b, c), being *Aspergillus flavus* and *Aspergillus fumigatus* two of them. Therefore, the effects of possible interactions need to be considered in the risk assessment process (Sexton and Hattis 2007).

Conclusions

It was possible to characterize the contamination caused by *Aspergillus flavus* complex and *Aspergillus fumigatus* complex in the seven poultry units using conventional and molecular methodologies. This study shows complementarily the cultural and molecular methods in the assessment of occupational exposure to fungi and raises the concern of occupational threat due to the detected fungal load, but also to the toxigenic potential of these species.

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