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# Mycobiota and mycotoxins present in finished fish feeds from farms in the Rio de Janeiro State, Brazil

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**Abstract**

The aim of the present study was to determine species of the fungal genera *Aspergillus*, *Fusarium*, and *Penicillium* and fumonisin $B_1$ ($FB_1$), aflatoxin $B_1$ ($AFB_1$), and ochratoxin A (OTA) contamination from feed intended for fish farms. A total of 60 samples were sampled from tilapia farms in the Rio de Janeiro State, Brazil. The quantitative enumeration of fungi as colony-forming units per gram of feed (CFU/g) was performed using the surface spread method in different culture media. The results were expressed as fungal isolation frequency and relative density. Fungal total counts ranged from $<1 \times 10^2$ to $4.7 \times 10^4$ CFU/g. *Fusarium* counts were not observed. Among toxigenic genera, *Aspergillus* (68%) was the most prevalent, followed by *Penicillium* species (60%). *Aspergillus niger* aggregate (36%), *Aspergillus flavus* (35%), and *Penicillium citrinum* (71%) were the most prevalent species. A high percentage of samples (98%) were contaminated with $FB_1$ levels, while 55% and 3.3% were contaminated with $AFB_1$ and OTA, respectively. The simultaneous occurrence of these mycotoxins emphasizes the need for further research in the area to better assess the risk to the health of fish farms and their implications for the health of consumers of this meat.

**Keywords:** Aflatoxin $B_1$, Fish feed, Fumonisin $B_1$, Mycobiota, Ochratoxin A

## Background

Aquatic products represent an important food source for animal and human consumption, the rising demand of which had led to a fast development of aquaculture (Myhr and Dalmo 2005). The primary objective in fish nutrition is to provide a nutritionally balanced mixture of ingredients as finished feed to support the maintenance, growth, reproductive performance, flesh quality, and health of the animals at an acceptable cost (NRC 1993). The quality of the products used in feeds for farmed fish has become a limiting factor for activity because these feeds are ideal substrates for the growth of fungi, which, under favorable conditions, may favor the synthesis of mycotoxins. Production of these toxic metabolites can occur during the growth of the crop, during post-harvest storage, or during the storage of the compounded feed (CAST Council for Agricultural Science and Technology 2003). The use of plant-based ingredients, such as corn, in aquafeeds enhances both the risk of introducing mycotoxins into the

feed at the point of feed manufacturing and mycotoxin production during storage of compounded feed (Spring and Fegan 2005).

Fumonisin is of concern to the aquaculture industry because it commonly contaminates corn and its by-products. There are several publications on the toxic effects of fumonisin $B_1$ ($FB_1$) in different fish species (Yildirim et al. 2000; Nguyen et al. 2003; Wang et al. 1991). Aflatoxin was the first of the mycotoxins to be investigated in aquaculture. As in other animal species, aflatoxin exerts carcinogenic effects in fish (Spring and Fegan 2005). Different researchers demonstrated the presence of aflatoxin $B_1$ ($AFB_1$) in shrimp and fish feed (Bautista et al. 1994; Abdelhamid et al. 1998). Although ochratoxin A (OTA) has not been studied to the same extent as $AFB_1$ in aquaculture, there are several studies demonstrating the toxic effects of this toxin in different fish species (Manning et al. 2003; Shalaby 2004; Moussa and Khattab 2003).

The number of studies addressing the effects of aflatoxins in aquatic species is very limited. The difficulty in accurately diagnosing aflatoxicosis in fish may in part explain the lack of information regarding the incidence of aflatoxicosis in farmed aquatic species. Initial findings associated with aflatoxicosis in fish include pale gills, liver damage, poor growth rates, and immune suppression. In fish, the role of fumonisins as toxic agents remains unclear. On one hand, minimal adverse effects have been reported in channel catfish fed with *Fusarium verticillioides* culture material containing 313 ppm of $FB_1$ for 5 weeks (Brown et al. 1994). On the other hand, for the same fish species, dietary levels of $FB_1$ of 20 ppm or above have been shown to result in lower weight gain and significant decrease in hematocrit and red and white blood cells than those at lower doses (Lumlertdacha et al. 1995). In rainbow trout, pathological signs of ochratoxicosis included liver necrosis, pale and swollen kidneys, and high mortality (Hendricks 1994).

Continuous studies regarding the monitoring of these toxins in finished feed to be used as animal feed are being performed worldwide (Magnoli et al. 2002; Akande et al. 2006; Keller et al. 2007; Glenn 2007; Martins et al. 2008; Cavaglieri et al. 2009; Pereyra et al. 2011). However, there is little information on feed contaminated with $FB_1$, $AFB_1$, and OTA intended for fish feed. The aim of the present study was to determine species of the fungal genera *Aspergillus*, *Penicillium*, and *Fusarium* and $FB_1$, $AFB_1$, and OTA contamination from finished feed intended for fish farms.

## Methods

### Sample source

Finished feed samples were collected from two tilapia farms. The samples came from the most important region of the Rio de Janeiro State where aquaculture practice is developed. The farms were representative as much in size and composition as in the kind of storage method applied. A total of 60 pelleted samples were sampled between September 2009 and August 2010. To ensure a correct sampling, each bag of 25 kg had a linear imaginary division in its length into three equal parts from which primary samples (1 kg) from the upper layer, central layer, and lower layer were collected. Samples were properly packed in bags and immediately sent to the laboratory. Samples were immediately processed for physical and mycological analyses and kept at –4°C until $FB_1$, $AFB_1$, and OTA analyses.

### Physical properties of samples

Water activity ($a_W$) determinations were carried out using AQUALAB CX2 (Decagon, Devices, Inc., Pullman, WA, USA). The device was calibrated using appropriate saline solutions in the work interval - according to procedures manual - before sample measurements.

### Mycological analysis

The quantitative enumeration of fungi as colony-forming units per gram of feed (CFU/g) was performed using the surface spread method described by Pitt and Hocking (1997). Ten grams of each sample was homogenized in 90 mL of distilled water solution for 30 min in an orbital shaker. Serial dilutions ($10^{-2}$ to $10^{-4}$) were made, and 0.1-mL aliquots were inoculated in triplicate onto the media dichloran rose bengal chloramphenicol agar (DRBC) for estimating total culturable fungi (Abarca et al. 1994) and dichloran 18% glycerol agar (DG18) that favors xerophilic fungus development. The plates were incubated at 25°C for 5 to 7 days. All samples were also inoculated onto Nash and Snyder agar to enumerate *Fusarium* species (Nelson et al. 1983). Nash-Snyder plates were incubated at 24°C for 7 days under a 12-h cold white/12-h black fluorescent light photoperiod. Only plates containing 10 to 100 CFU were used for counting, with results expressed as CFU/g of sample. On the last day of incubation, individual CFU/g counts for each colony type considered to be different were recorded. Colonies representative of *Aspergillus* and *Penicillium* were transferred for sub-culturing to tubes containing malt extract agar, whereas *Fusarium* spp. were transferred for sub-culturing to plates containing carnation leaf agar.

Species of *Aspergillus*, *Penicillium*, and *Fusarium* were identified according to Klich (2002), Samson et al. (2000), and Nelson et al. (1983), respectively. The results were expressed as isolation frequency (percentage of samples in which each genus was present) and relative density (percentage of isolation of each species among the same genus).

### Fumonisin $B_1$ detection and quantification

A commercially available enzyme-linked immunosorbent assay plate kit, AgraQuant® Total Fumonisin Assay (Romer Labs®, Campinas, Brazil), was applied for the extraction and quantification of $FB_1$. Mycotoxin extraction and testing were performed according to the manufacturer's instructions. A 20-g portion of each sample was extracted with 100 mL of methanol for 3 min into a blend jar. The mixture was diluted in water (1:20 *v*/*v*) and an aliquot taken and placed into a culture plate. Detection limit of the technique was 0.2 μg/g.

### Aflatoxin $B_1$ and ochratoxin A determination

The extraction of $AFB_1$ and OTA was determined according to Soares and Rodriguez-Amaya (1989). Detection and quantification evaluation was made by thin-layer chromatography. Detection limits of the used techniques were 0.003 and 0.005 μg/g for $AFB_1$ and OTA, respectively.

### Statistical analyses

Data analyses were performed by analysis of variance. Data on total fungal counts were transformed using the logarithmical function $\log_{10}(x + 1)$ before applying the analysis

of variance. The LSD test was used to determine the significant differences between means. The analysis was conducted using the BioEstat 5.0 program.

## Results

### Water activity content

The fish feed samples tested had $a_W$ mean values of 0.598 ± 0.053.

### Mycological isolation and identification

Fungal total counts on the DRBC and DG18 media ranged from $<1 \times 10^2$ to $4.7 \times 10^4$ and from $< 1 \times 10^2$ to $2.7 \times 10^4$ CFU/g, respectively. The mean fungal total counts were $2.9 \times 10^3 \pm 0.6 \times 10^3$ (medium ± standard deviation (SD)) in DRBC and $2.2 \times 10^3 \pm 3.8 \times 10^3$ (medium ± SD) in DG18 medium. *Fusarium* spp. were not present on the SNA medium.

Based on the frequency of fungal genera (%), eight filamentous genera were obtained. *Cladosporium* (85%) was predominant. Among the toxigenic genera, the genus *Aspergillus* (68%) was the most prevalent, followed by *Penicillium* species (60%). Other genera were isolated at lower frequencies such as *Wallemia*, *Eurotium*, *Aureobasidium*, *Mucor*, and *Nigrospora* species (Figure 1).

Figure 2 shows the relative density (%) of *Aspergillus* spp.; *Aspergillus niger* aggregate and *Aspergillus flavus* were the most prevalent, followed by *Aspergillus versicolor*. *Aspergillus fumigatus*, *Aspergillus candidus*, and *Aspergillus oryzae* were found at low relative densities.

Figure 3 shows the relative density (%) of *Penicillium* spp. A high diversity of *Penicillium* species was found. *Penicillium citrinum* was the most prevalent, followed by *Penicillium glabrum*.

### Fumonisin $B_1$ determination

Samples contaminated with $FB_1$ levels (90%) ranged from 0.3 to 4.94 μg/g and 2.6 μg/g as medium level.

### Aflatoxin $B_1$ and ochratoxin A

Fifty-five percent and 3.3% of samples were individually contaminated with $AFB_1$ and OTA, respectively, in levels detectable but not quantifiable for the technique used.

### Co-occurrence

Of total samples proved to have $AFB_1$ (55%) and $FB_1$ (98%), 50% showed co-occurrence of these mycotoxins. The 3.3% of the samples presented co-occurrence of the three mycotoxins analyzed.

## Discussion

The mycobiota and mycotoxin contamination present in finished feeds intended for fish was studied. In this study, moderate levels of colony counts were found. These results were similar to those obtained in feed from marine shrimp studied in Piaui State (Santos 2006). However, in our study, 10% of samples analyzed exceeded the levels proposed as hygienic feed quality limits in DRBC ($1 \times 10^4$ CFU/g; Good Manufacture

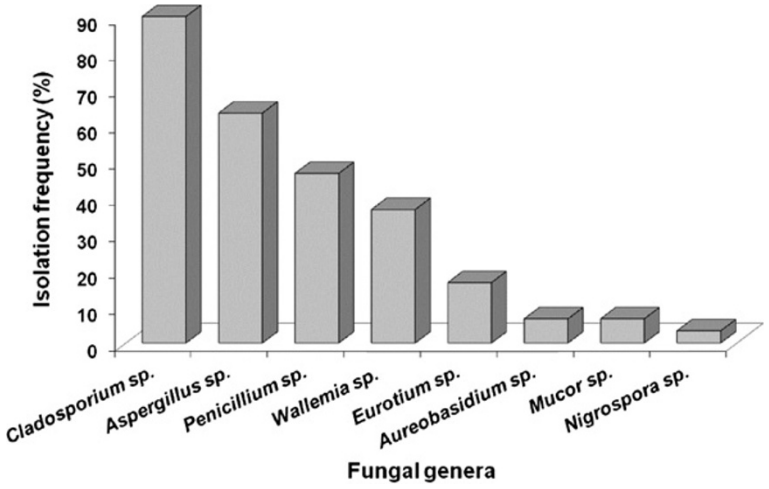


**Figure 1** Frequency (%) of fungal genera isolated from feeds intended for fish farm.

Practice 2008). The DRBC medium, used to estimate total culturable fungi (Abarca et al. 1994), and DG18, which favors xerophilic fungus development, revealed high counts of mold, and this corresponds to dry feed as is being studied here.

Fungal growth leads to the reduction of the nutritional quality of fish feed samples that could affect the palatability of feed and reduce the animal's nutrient absorption, determining a low-quality substrate and an improper storage. Little published information about fungal contamination in feeds for aquaculture in Brazil has been found. Cardoso (2011) obtained counts over the proposed limits in fish feed samples in the north of Brazil, reporting high counts in 56% and 11% of samples from juvenile and growing stages, respectively. Nunes (2009), who also studied fish feed, showed that 67% feed samples had counts above the proposed limit by Good Manufacture Practice (2008).

Many studies have shown that most feeds have species of the genera *Aspergillus* and *Penicillium* as predominant in pelleted feed (Keller et al. 2007, 2008; Pereyra et al. 2009; Fernández Juri et al. 2009). Our results showed that *Aspergillus* and *Penicillium* species had the highest isolation frequencies. These results are similar to those obtained

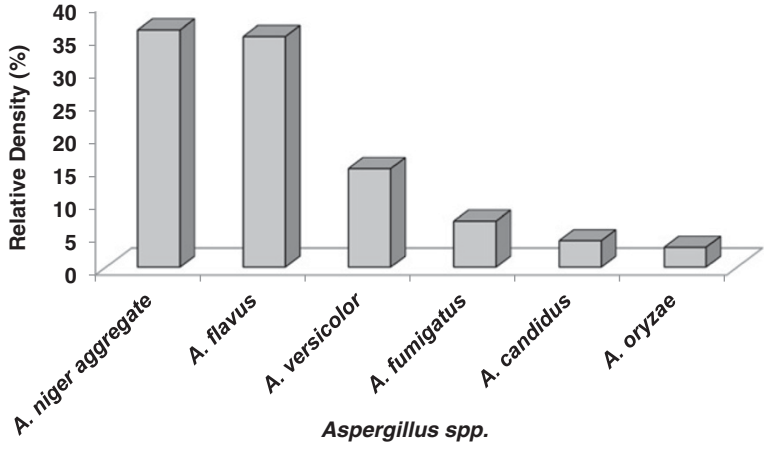


**Figure 2** Relative density (%) of *Aspergillus* species in feeds intended for fish farm.

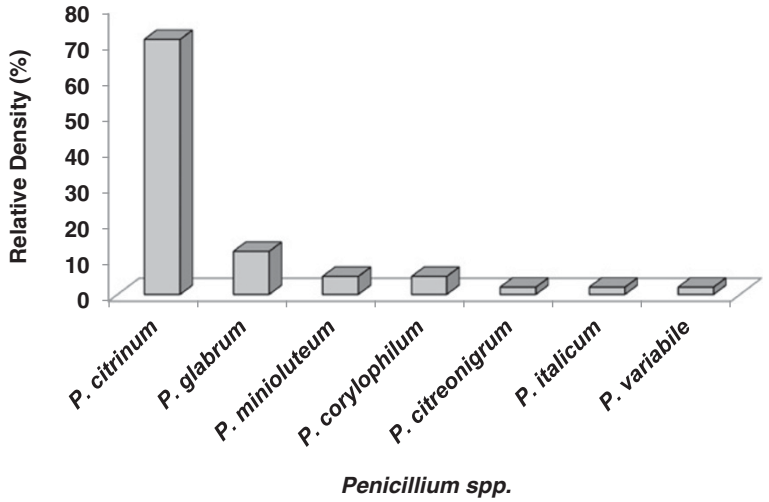


**Figure 3** Relative density (%) of *Penicillium* species in feeds intended for fish farm.

by Santos (2006) and Cardoso (2011) in marine shrimp and fish feed samples. However, these authors also reported *Fusarium* species in fish feed samples.

In this study, *A. niger* aggregate and *A. flavus* were the most prevalent species isolated from these types of samples, followed by *A. versicolor*. Santos (2006), Nunes (2009), and Cardoso (2011) reported high percentages of *A. flavus* in feed samples from aquaculture in the north of Brazil. Other studies found *Aspergillus* section *Flavi* species followed by *A. niger* aggregate from equine, poultry, and pet pelleted feed samples as prevalent (Keller et al. 2007; Pereyra et al. 2009; Fernández Juri et al. 2009).

Many of the *Penicillium* species found (*P. citrinum*, *P. glabrum*, *P. minioluteum*, *P. citreonigrum*, *P. corylophilum*, *P. italicum*, and *P. variabile*) can produce a very wide range of toxic compounds such as citrinin and citreoviridin (Pitt 2004). So far, there is no information about the toxicological effects of these mycotoxins in fish.

Some researchers have stressed the importance of fungal determination in order to provide information about which mycotoxin could be present (Dalcero et al. 1997, 1998; Magnoli et al. 2002). In this work, the frequency of *A. flavus* and $AFB_1$ contamination showed a positive relation, whereas both *A. niger* aggregate with OTA and *F. verticillioides* with $FB_1$ contamination showed a negative relation. In this work, *F. verticillioides* strains were not isolated; however, levels of $FB_1$ in 98% of feed samples were found. *Fusarium* is a genus of field fungi, of which species do not have the ability to grow in dry feed, but it is probable that $FB_1$ has been produced in the field and comes with the raw material that composes the finished feed.

The pelleted ones are produced in a humid process with heat, where the product reaches a temperature between 85°C and 90°C, with 15.5% to 17% of humidity, for 30 to 45 s. Considering the process of feed elaboration, where the feed is subjected to high temperatures, the inactivation of fungal propagules could be expected and not $FB_1$, which is inactivated at 150°C (WHO World Health Organization 2001).

*Fusarium* toxins are important not so much for their acute effects as for the chronic syndromes reported worldwide. Dietary fumonisin at 20 ppm has been shown to reduce growth rate in catfish with an average weight of 1.5 g (Yildirim et al. 2000). Two-year -old catfish only had reduced weight gain when exposed to 80 ppm of fumonisin. Toxic

effects of fumonisin have been reported in the Nile tilapia at concentrations similar to those in catfish (Nguyen et al. 2003). A survey of catfish feed ingredients in Alabama and Mississippi in the USA revealed that 80% of the corn samples contained detectable levels of fumonisin in concentrations that ranged from 1.3 to 10 ppm (Lumlertdacha and Lovell 1995). In this work, the principal ingredient in the fish feed also was the corn. In our study, $FB_1$ levels in the fish feed samples did not exceed the permissible limit from animal feed (5 ppm; Good Manufacture Practice 2008). However, chronic levels could cause adverse effects in fish. According to the European Union recommendations (2003 and 2006), the tolerable limits for fumonisins ($FB_1$ + $FB_2$), $AFB_1$, and OTA are 10, 0.02, and 0.25 ppm, respectively, in animal feed (except dairy cows for $AFB_1$).

The presence of $AFB_1$ in this work shows a frequency higher than that found by Hashimoto et al. (2003) in feed used for aquaculture in the region of Londrina, Paraná, which was 28.5% of total aflatoxin-contaminated samples, $AFB_1$ being the principal. In another study of commercial feeds for fish in the north and west of the state of Paraná, Buck (2005) detected total aflatoxin contamination in 17% of samples. The frequency contamination of OTA is similar to that found by Buck (2005), where 4% of feed samples analyzed showed only OTA.

Exposure to mycotoxins often occurs simultaneously. The present study has shown the simultaneous occurrence of three carcinogenic mycotoxins, $FB_1$, $AFB_1$, and OTA, in feed intended for fish farms. There are no studies published on feed contamination simultaneous with these three toxins intended for fish farms in Brazil.

The biological effects of mycotoxins depend on the ingested amounts, number of occurring mycotoxins, time of exposure, and animal sensitivity. Moreover, the mycotoxins' effects are not only amplified by stress production but also increased in intensively reared animals (Yiannikouris and Jouany 2002; Binder 2007).

## Conclusions

The simultaneous occurrence of $FB_1$, $AFB_1$, and OTA emphasizes the need for further research in the area to better assess the risk to the health of fish farms and their implications for the health of consumers of this meat. The first important step in controlling the fungal and mycotoxin contamination in finished feed is to control them in the raw materials from which the feed is prepared in order to prevent the occurrence of mycotoxicosis in aquaculture, to reduce economic losses, and to minimize hazards to human health.

#### Competing interests

The authors declare that they no competing interests.

#### Authors' contributions

TB carried out the mycological and mycotoxin analysis. CP was responsible for the organization of the results and drafted the manuscript. CS participated in the mycological analysis. ED carried out the sampling and analysis of physical properties of samples. AO participated in the mycotoxin analysis. KK carried out the statistical analysis and coordination of the study. PS participated in the design of the study. LC was responsible for the organization of the results and editing of the manuscript. CR carried out the design and coordination of the study. All authors read and approved the final manuscript.

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