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Short Communication

Screening of aflatoxin B_1 and mycobiota related to raw materials and finished feed destined for fish

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ABSTRACT. The aim of the present study was to determine fungal genera, *Aspergillus*, *Penicillium* and *Fusarium* species and aflatoxin B_1 contamination from raw materials and finished feed intended for fish farm localized in Piaui, Brazil. *Aspergillus flavus* and *P. citrinum* were isolated with a high relative density from all samples. In general, a high percent of samples exceeded the levels proposed as feed hygienic quality limits (CFU g^{-1}) according to Good Manufacture Practice. Aflatoxin B_1 was analyzed by enzyme-linked immunosorbent assay. All raw materials and finished feed showed aflatoxin B_1 levels. Although in this study AFB_1 levels below recommended limits (20 µg kg^{-1}) were found, it is important to emphasize the feed intake with toxin in low concentrations along time, since it produce chronic deleterious effects in animal production. This fact requires periodic monitoring to prevent the occurrence of chronic aflatoxicosis in aquaculture, to reduce the economic losses and to minimize hazards to animal health.

Keywords: aflatoxin B_1, mycobiota, finished feed fish, raw materials, aquaculture.

Monitoreo de aflatoxina B_1 y micobiota relacionada a materias primas y alimentos terminados destinados a peces

RESUMEN. El objetivo de este estudio fue determinar géneros fúngicos, especies de *Aspergillus*, *Penicillium* y *Fusarium* y la contaminación de aflatoxina B_1 (AFB_1) en materias primas y alimento terminado destinado a la cría de peces en el estado de Piauí, Brasil. *Aspergillus flavus* y *P. citrinum* fueron aisladas con alta densidad relativa. En general, un alto porcentaje de muestras excedieron los niveles fúngicos propuestos como límite de calidad higiénica (CFU g^{-1}) según las buenas prácticas de manufactura. Aflatoxina B_1 fue analizada por ensayos inmuno-enzimáticos. Todas las materias primas y el alimento terminado mostraron niveles de contaminación con AFB_1. Aunque en este estudio los niveles de AFB_1 estuvieron por debajo del límite recomendado (20 µg de toxina kg^{-1} de alimento), es importante enfatizar la ingesta de alimento con bajas concentraciones de toxina a lo largo del tiempo ya que produce efectos crónicos en animales de producción. Este factor requiere monitoreo periódico para prevenir la incidencia de aflatoxicosis crónica en acuacultura, reducir las pérdidas económicas y minimizar los riesgos de la salud animal.

Palabras clave: Aflatoxina B_1, micobiota, alimento terminado, materia prima, acuicultura.

Aquaculture is currently the fastest growing animal production sector in the world. Brazil obtained the third position in production of aquaculture in America (FAO, 2011; MPA, 2011). This increase in aquaculture production must be supported by a corresponding increase in the production of formulated diets for the cultured aquatic animals. For most aquaculture systems, the cost of feed constitutes 30 to 60% of the farm operational costs (Wing-Keong, 2003). The improvement in the formulation and preparation of diets for fish requires the

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detection of factors that negatively affect the quality of feed, to avoid production losses (Conroy, 2000). Mycotoxins are fungal secondary metabolites associated with severe toxic effects to vertebrates and produced by important fungi including *Aspergillus, Penicillium, Fusarium,* and *Alternaria* species (Kabak *et al*., 2006). Aflatoxins (AFs) are a group of naturally occurring mycotoxins produced by *Aspergillus* fungi, especially *A. flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1), in particular, is toxic to many species of animals, as well as humans, and presents carcinogenic, teratogenic and mutagenic potential (IARC 2002, Sassahara *et al*., 2005). There are many reports on the contamination of mycotoxins in various raw materials and feeds for animals (Akande *et al*., 2006; Glenn, 2007; Keller *et al*., 2007; Martin *et al*., 2008; Cavaglieri *et al*., 2009; Pereyra *et al*., 2011). Several reports on evidence of the negative impact of mycotoxins in fish species were informed in the south of Brazil (Lopes *et al*., 2005, 2010; Vieira *et al*., 2006). However, there is no published information on feed contaminated with fungal genera and AFB_1 intended for fish feed in northeast of Brazil. The aim of the present study was to determine fungal genera, *Aspergillus*, *Penicillium* and *Fusarium* species and AFB_1 contamination from raw materials and finished feed intended for fish.

Raw materials and finished fish feed samples were collected from one industry in Piauí State, Brazil (5º5′20″S, 42°48′7″W). This industry was selected because it is the major provider of feed intended for fish farm in the northeast of Brazil. A total of 18 raw materials and 36 finished fish feed samples (pelleted) were sampled from January to March 2009. 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 k) from the upper layer, central layer and lower layer were collected. Three kinds of raw materials: soybean bran, corn bran, and other cereals (wheat meal, fishmeal and meat meal) in small amount intended for feed manufacturing were collected. The composition of the finished feed samples was composed of soybean bran (15%), corn bran (27%), other cereals (57.5%), and vitamin-mineral mix (0.5%). Samples were properly packed in bags and immediately sent to the laboratory. Samples were immediately processed for mycological analyses and kept at -4°C until AFB_1 analyses.

Analysis of the mycobiota was made by the plate dilution spread method onto dichloran rose bengal chloranphenicol agar (DRBC), a general medium used for estimating total mycobiota (Abarca *et al.,* 1994). Quantitative enumeration was done using the surface-spread method. Twenty-five grams of each sample were homogenized in 225 mL 0.1% peptone water solution for 30 min in an orbital shaker. Serial dilutions (10^{-2} to 10^{-3}) were made and 0.1 mL aliquots were inoculated in duplicates onto the culture media. Plates were incubated at 25ºC for 7 days in darkness. Only plates containing 10-100 colony-forming units (CFU) were used for counting. The results were expressed as CFU g^{-1} per sample. Representative colonies of *Aspergillus* and *Penicillium* were transferred for sub-culturing to tubes containing malt extract agar (MEA) whereas *Fusarium* spp. were transferred for sub-culturing to plates containing carnation leaf agar (CLA). 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 (% of samples in which each genera was present) and relative density (% of isolation of each species among strains of the same genera).

A commercially available enzyme-linked immunosorbent assay (ELISA) plate *Kit* AgraQuant® Total Aflatoxin Assay (Romer Labs®) was applied for the extraction and quantification of AFB_1. A 20 g portion of each sample was extracted with 100 mL methanol during 3 min into a blend jar. The mixture was diluted in water (1:20 v v^{-1}) and an aliquot taken and placed into a culture plate. Detection limit of the technique was 1.0 µg kg^{-1} (ppb) for AFB_1.

Data analyses were performed by analysis of variance. Total fungal counts data were transformed using a logarithmical function $\log_{10}$ (x+1) before applying the analysis of variance. The Student-Newman-Keuls (SNK) test was used to determine the significant differences between means. The analysis was conducted using SIGMA STAT program.

Table 1 show the fungal counts from raw materials and finished fish feed in DRBC culture media. In general, a high percent of raw materials and finished feed samples had counts higher than $1x10^4$ CFU g^{-1}.

A mycological survey of the samples indicated the presence of nine genera of filamentous fungi (Table 2). Table 3 shows the relative density of isolated of *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. isolated from raw materials and finished fish feed. *Aspergillus flavus* and *P. citrinum* were isolated with a high relative density from all raw materials and finished fish feed. *Aspergillus parasiticus* was isolated from all raw materials but not of finished fish feed. *Aspergillus section nigri* (*A. carbonarius* and *A. niger* aggregate) strains were isolated of soybean bran and other cereals with a relative density from 23.1 and 16.7%, respectively. *Aspergillus versicolor, A. fumigatus* and *A. terreus* strains were isolated at lower relative densities. *Penicillium pinophylum* was isolated only in corn bran with a relative density of 35% and *P. funicu-*

Table 1. Fungal counts (CFU g^{-1}) from raw materials and finished fish feed in DRBC culture media. DRBC: dichloran rose bengal chloranphenicol, detection limit: $1x10^2$ CFU g^{-1}, maximum recommended level: $1x10^4$ CFU g^{-1} (GMP, 2008). (*) % samples exceeded the maximum recommended level, a = the same letters represent similar results ($P > 0.05\%$).

Samples		Fungal counts (CFU g^{-1}) Media-range	Samples over limits (%)*
Raw materials samples	Soybean bran	$3.23x10^{4\,a}$ ($9.33x10^3$ - $5.62x10^4$)	83.3
	Corn bran	$6.92 \times 10^{4\,a}$ ($1.55x10^4$ - $2.45x10^5$)	100
	Other cereals	$3.72 \times 10^{3\,b}$ ($<1.0x10^2$ - $3.47x10^4$)	66.7
Finished feed samples		$2.40 \times 10^{4\,a}$ ($4.47x10^3$ - $1{,}02x10^5$)	66.7

Table 2. Isolation frequency of fungal genera (%) from raw materials and finished fish feed.

Fungal genera	Isolation frequency (%)			
	Raw materials samples			Finished feed samples
	Soybean bran	Corn bran	Other cereals	
Penicillium sp.	100	100	66.7	83.3
Aspergillus sp.	83.3	66.7	66.7	66.7
Fusarium sp.	0	16.7	0	0
Cladosporium sp.	0	0	0	20
Paecilomyces sp.	16.7	0	0	0
Acremonium sp.	0	16.7	0	0
Byssochlamys sp.	16.7	0	0	0
Geotrichum sp.	0	0	16.7	0
Rizhopus sp.	16.7	16.7	16.7	23.3

losum was present in the soybean bran and corn bran. *Penicillium purpurogenum*, *P. corylophilum*, *P. miczynskii*, *P. implicatum* and *P. variable* strains were isolated at lower relative densities. *Fusarium verticillioides* was only present in corn bran.

Table 4 show the AFB_1 levels in raw materials and finished fish feed. Aflatoxin B_1 was detected in mean levels of 5.8 µg kg^{-1} (soybean bran), 1.1 µg kg^{-1} (corn bran), 7.4 µg kg^{-1} (other cereals) and 3.8 µg kg^{-1} (finished fish feed) with a frequency from 40, 33.3, 60 and 16.7%, respectively.

Fungi and AFB_1 contamination from raw materials and finished feed intended for fish farm were studied.

The quality of food is an essential prerequisite for obtaining optimal production results in fish production (Jakic-Domic *et al.*, 2005). Fungal growth leads to reduction of the nutritional quality of the raw materials, and may contribute to the contamination of the finished fish feed by fungi (Cavaglieri *et al.*, 2009). In this study, a high mycological contamination in raw materials and finished feed was found. The collected soybean bran (83.3%), corn bran (100%), other cereals (66.7%) and finished fish feed (66.7%) samples exceeded the limit $1x10^4$ CFU g^{-1} that determines feed hygienic quality, according to good manufacturing practices (GMP, 2008). These results suggest a high fungal activity that could affect the palatability of feed and reduce the animal nutrients absorption, determining a low quality substrate (Ogundero, 1987; Martins & Martins, 2001). The screening of samples for fungal propagules is a useful exercise in itself as an indicator of contamination but also complements the analysis of mycotoxins that could be present.

High fungal diversity was found in raw materials and finished fish feed. All samples showed that *Aspergillus* spp. and *Penicillium* spp., the main toxigenic fungus, were the prevalent genera. Many studies have shown that most feed have species of *Aspergillus* and *Penicillium* genera as predominant in pelleted or extruded feed (Keller *et al.*, 2007, 2008; Fernandez-Juri *et al.*, 2009; Pereyra *et al.*, 2009). Our results are similar to those obtained by Santos (2006) and Calvet (2008) in marine shrimp and Cardoso (2011) in finished fish feed samples. However, these authors did not analyze the raw materials.

Table 3. Relative density (%) of *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. isolated from raw materials and finished fish feed.

Species	Relative density (%)			
	Raw materials samples			Finished feed samples
	Soybean bran	Corn bran	Other cereals	
A. flavus	61.5	50	66.6	100
A. parasiticus	7.7	25	16.7	0
A. versicolor	7.7	8.3	0	0
A. fumigates	0	8.3	0	0
A. terreus	0	8.3	0	0
A. carbonarius	7.7	0	16.7	0
A. niger aggregate	15.4	0	0	0
P. citrinum	81.8	35	90.9	94.1
P. purpurogenum	0	0	0	5.9
P. pinophylum	0	35	0	0
P. funiculosum	9.1	20	0	0
P. corylophilum	0	0	9.1	0
P. miczynskii	0	5	0	0
P. implicatum	0	5	0	0
P. variabile	9.1	0	0	0
F. verticillioides	0	100	0	0

Table 4. Aflatoxin B_1 levels in raw materials and finished fish feed. †Contamination frequency (%): percentage of samples contaminated with AFB_1.

Mycotoxins		Raw material samples			Finished feed samples
		Soybean bran	Corn bran	Other cereals	
AFB_1	Media ± range (μg kg^{-1})	5.8 (1.3-10.3)	1.1 (1.0-1.7)	7.4 (1.5-19.1)	3.8 (1.6-9.8)
	Frequency† (%)	40	33.3	60	16.7

In this study, a high frequency of *Aspergillus* species was found. *Aspergillus flavus* and *A. parasiticus* were the predominant species isolated. This species are important aflatoxins producers, mainly AFB_1 (CAST, 2003). Santos (2006), Calvet (2008) and Cardoso (2011) reported high percentages of *A. flavus* in feed samples from aquaculture in northeast of Brazil. Other studies found *Aspergillus* section *Flavi* species from equine, poultry and pet-pelleted feed samples as prevalent (Keller *et al*., 2007; Fernandez-Juri *et al*., 2009; Pereyra *et al*., 2009). Many of the *Penicillium* species found (*P. citrinum*, *P. purpurogenum*, *P. pinophylum*, *P. funiculosum*, *P. corylofhilum*, *P. miczynskii*, *P. implicatum* and *P. variabile*), can produce a very wide range of toxic compounds such as citrinin and cetroeviridin (Pitt, 2004). So far, there is no information about the toxicological effects of these mycotoxins in fish. In this study, *F. verticillioides* was found only in corn bran. Other authors reported *Fusarium* species in marine shrimp and fish feed samples (Santos, 2006; Cardoso, 2011). *Fusarium* is a genus of field; these species do not have the ability to grow in dry feed such as pelleted and extruded feed.

All raw materials and finished feed showed aflatoxin B_1 levels. This result agrees with those obtained by Hashimoto *et al*. (2003) in feed used for aquaculture in the region of Londrina, Paraná, which was 28.5% of total aflatoxins contaminated samples, being AFB_1 the principal. In other study of commercial feeds for fish in the north and west of the State of Parana, Buck (2005) detected total aflatoxins contamination in 17% of samples with levels of 7.84 to 26.49 μg kg^{-1}.

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

in this study AFB_1 levels below recommended limits (20 µg kg^{-1}, GMP, 2008) were found, it is important to emphasize the feed intake with toxin in low concentrations along the time since produce chronic deleterious effects in animal production. El-Sayed & Khalil (2009) described that a prolonged feeding of European seabass with low levels of AFB_1 (1.8 µg kg^{-1} body weight) causes not only serious health problems in exposed-fish, but also represents a high risk to consumers through AFB_1 residues in fish musculature. This might also pose human health concerns as certain aflatoxin levels were recorded in the muscles of fish fed contaminated diets. Han *et al*. (2009) described that gibel carp, *Carassius auratus gibelio* (L.) fed with more than 10 µg AFB_1 kg^{-1} diet showed accumulation of AFB_1 residues in muscles and ovaries above the safety limitation of European Union (2 ppb).

It is very difficult to guarantee the absence of mycotoxins in aquaculture feeds even when appropriate measures are taken, such as good screening programs, selection of high quality raw materials and feed ingredients, and good storage conditions. It is therefore imperative to find effective ways of managing the risks posed by mycotoxin contamination.

This is the first report that provides information on the fungal and AFB_1 contamination in raw materials and finished feed intended for fish farm localized in Piauí, Brazil. Future studies could be conducted to analyze other mycotoxins, as fuminisins and citrinin.

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