

## Toxigenic mycobiota and mycotoxins in shrimp feed

### Micobiota tóxigena e micotoxinas em ração de camarão

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#### ABSTRACT

The objective of this study was to identify the toxigenic mycobiota and the occurrence of aflatoxins in shrimp feed products intended for shrimp cultivated in the coastal area of the state of Piauí, Brazil, in three farms ("A", "B" and "C"). The toxigenic capacity of the fungal species isolated was tested for aflatoxins (AF) and ochratoxin A production. The fungal counts of shrimp feed were similar for the "A" and "B" farms at all cultivation phases, collection sites, in closed and opened packages (1.33 to 2.66CFU g<sup>-1</sup> log<sub>10</sub><sup>-1</sup>). The lowest fungal counts were found in feed from "C" farm (0.65CFU g<sup>-1</sup> log<sub>10</sub><sup>-1</sup>) from closed packages. Thirty-four strains of *Aspergillus* were detected with a greater prevalence of *A. flavus*. Two strains produced B1, B2, G1 and G2 aflatoxins at concentrations from 0.39 to 0.42ng g<sup>-1</sup>; 0.18 to 0.27ng g<sup>-1</sup>; 1.78ng g<sup>-1</sup> and 0.09ng g<sup>-1</sup> respectively and were classified as atypical *A. flavus*. Two strains of *A. niger* aggregate were OTA producers. Fifteen samples (13.88%) presented AFB1 contamination at levels ranging from 0.25ng to 360ng g<sup>-1</sup>. This study demonstrates the presence of toxigenic fungi in shrimp feed used at different phases of cultivation and farms. Atypical strains of *A. flavus* were isolated which produced AF B1, B2, G1 and G2 in shrimp feeds. Only AFB1 was detected in the analyzed feed.

**Key words:** *Litopenaeus vannamei*, feed storage, toxigenic fungi, mycotoxins, aflatoxin B1.

#### RESUMO

O objetivo deste estudo foi identificar a micobiota toxigênica e a incidência de aflatoxinas em rações comerciais para camarão cultivado no litoral do Estado do Piauí, Brasil, em três fazendas ("A", "B" e "C"). Foi realizada a capacidade toxigênica das espécies de fungos isolados e a produção de aflatoxinas (AF) e ocratoxina A (OTA). As contagens fúngicas da ração foram semelhantes nas fazendas "A" e "B" e em todas as fases de cultivo, locais de coleta e de embalagens fechadas e abertas (1,33-2,66UFC g<sup>-1</sup> log<sub>10</sub><sup>-1</sup>). As mais baixas contagens de fungos foram encontradas

nas rações de embalagens fechadas da fazenda "C" (0,65UFC g<sup>-1</sup> log<sub>10</sub><sup>-1</sup>). Foram isoladas trinta e quatro cepas de *Aspergillus* com maior prevalência de *A. flavus* e duas linhagens eram produtoras de aflatoxinas B1, B2, G1 e G2 em concentrações 0,39-0,42ng g<sup>-1</sup>; 0,18-0,27ng g<sup>-1</sup>; 1,78ng g<sup>-1</sup>, e 0,09ng g<sup>-1</sup>, respectivamente, e foi classificado como *A. flavus* atípico, sendo necessária posteriormente a classificação filogenética desta cepa. Duas cepas de *A. niger* agregados eram produtoras de OTA. Quinze amostras de ração (13,88%) apresentaram contaminação AFB1 em níveis que variam de 0,25ng a 360ng g<sup>-1</sup>. Este estudo demonstra a presença de fungos toxigênicos em rações de camarão nas fazendas analisadas e nas diferentes fases de cultivo. Foram isoladas, em rações de camarões, cepas atípicas de *A. flavus*, produzindo AF B1, B2, G1 e G2. Apenas AFB1 foi detectada na ração analisada.

**Palavras-chave:** *Litopenaeus vannamei*, alimentos armazenados, fungos toxigênicos, micotoxinas, aflatoxina B1.

#### INTRODUCTION

The global production of cultivated and captured shrimp was of 6,624,387 tons in 2006, with 47.77% from cultivation (FAO, 2008). Shrimp cultivation is an important activity in the coastal area of the state of Piauí. This activity generates foreign exchange and is an important source of employment for the local population (ROCHA, 2007). In 2006, the Brazilian production was of 65.000 tons of shrimp, with 843 tons being from shrimp farms in Piauí, ranking the 6<sup>th</sup> place nationally (ABCC, 2010).

In shrimp farming, feed is stored in warehouses and storage sites near the tanks to facilitate

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management under carefully controlled conditions, especially in humid and hot weather regions favoring the growth of contaminating organisms, such as coliforms, enterobacteria, fungi and yeast (FAO, 2008).

Many studies on fungal mycobiota in food and feed samples have reported the frequent presence of potentially toxigenic fungi. Mycotoxins are secondary metabolites secreted by moulds, mostly belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium*. Several moulds, capable of producing several toxins, frequently contaminate feeds simultaneously and have synergistic effects (GARCIA, et al., 2009).

Fungi are able to produce more than one mycotoxin, with some mycotoxins being produced by more than one fungal species. Therefore, several mycotoxins are often simultaneously found in a single product. Animal feed Contamination and potential contamination of their meat by mycotoxins are a serious hazard to humans and animals. (PEREYRA, et al., 2010). 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 (BARBOSA et al., 2013).

However, the incidence and relative importance of these different mycotoxins in animals have not yet been established. Studies show that aflatoxin B<sub>1</sub> is the most toxic of the aflatoxins and is a potent liver carcinogen. Substantial evidence also indicates that exposure to low levels of aflatoxins may suppress the immune system and increase susceptibility to diseases (CAST, 2003).

Among the mycotoxins, aflatoxins are extremely biologically active secondary metabolites produced by the fungi, *Aspergillus* species. These toxicants are particularly important in aquaculture since their presence exerts a negative economic impact on relevant commerce as well as severe health problems after exposure to infected food and feed. Toxic feed contaminants can cause abnormalities such as poor growth, physiological imbalances and histological changes that result in yield reduction and profitability of shrimp culture (GOPINATH, et al., 2012).

Aflatoxins are produced by strains of *Aspergillus flavus*, *A. parasiticus* and *A. nomius*, which can often grow in stored foods. Phylogenetic studies of *A. flavus* showed that it consists of two subgroups (I and II) (TRAN-DINH et al., 1999). Most group I strains produced Aflatoxin B, and most group II strains produced both Aflatoxin B and Aflatoxin G (PILDAÍN et al. 2004; PERRONE et al., 2007).

Factors depressing the immunological system of shrimp, combined with factors such as the presence of pathogens may cause a reduction in its survival rate or jeopardize the visual aspect of the final product (NUNES et al. 2004). When consuming aflatoxin B<sub>1</sub> contaminated food, *Litopenaeus vannamei* and *Penaeus stylirostris* presented lesions in the hepatopancrea, antennal gland, mandible organ and hematopoietic organs (BINTVIHOK et al., 2003). Levels of 1000 ng/g of aflatoxin B<sub>1</sub> also reduces shrimp growth; decreases blood cells, making shrimp more vulnerable to pathogens; causes decrease in lipid deposit by the hepatopancrea cells and reduction of the survival rate (BOONYARATPALIN et al., 2001; GOPINATH & RAJ, 2009, GOPINATH et al., 2012). The objective of this research was to quantify and identify the toxigenic mycobiota and aflatoxins occurrence in feed intended for shrimp cultivated in the coastal area of Piauí, Brazil.

## MATERIALS AND METHODS

### Samples

Three out of fourteen farms in the coastal area of Piauí, Brazil, were selected for this study, and denominated "A", "B" and "C". Each farm (A, B and C) had one tank for each cultivation phase (post larvae – phase I, juvenile–phase II, and fattening–phase III), on each one of this tank six samples were collected, totaling 108 feed samples of 1.0kg.

The experiment arranged in a 3x2 factorial scheme (types of ration and storage), with six repetitions, each represented by commercial feed samples of 1.0kg. Samples from closed and opened feed packages were collected at the storage warehouse and storage facilities near the tanks. The relative temperature and humidity of the environment were measured using a portable Inconterm<sup>®</sup> thermohygrometer. The samples were packed in sterile Nasco Whirl-Pak<sup>®</sup> plastic bags, appropriately identified and transported to the Food Microbiological Control Laboratory of the Center of Studies and Food Processing of the Universidade Federal do Piauí, Brazil.

### Mycobiota determination and identification of *Aspergillus* species

Total fungal count in the feed samples was conducted in Dichloran-Rose Bengal chloramphenicol agar (DRBC), a general medium used for estimating total cultivable mycobiota, recommended by PITT & HOCKING (2009). The results were expressed as CFU per gram of sample (CFU g<sup>-1</sup>). Representative colonies of *Aspergillus* spp. were transferred for sub-culturing into tubes containing malt extract agar (MEA). Identification of *Aspergillus* species was performed according to taxonomic keys (KLICH, 2002).

### Toxigenic capacity of *Aspergillus*

#### Aflatoxin production by *Aspergillus* section *Flavi*

All *Aspergillus* section *Flavi* strains isolated from ration was assayed for aflatoxin production. The strains were grown on MEA plates at 28°C for seven days. The mycelium was transferred to a tube and 1000µL chloroform was added. The mixture was shaken for 20min at room temperature, the mycelium was removed and the chloroform extract evaporated to dryness under N<sub>2</sub> flow. The residue was re-dissolved in 200µL of chloroform (GEISEN, 1996). The extracts were analyzed by High Performance Liquid Chromatography (HPLC) using a SHIMADZU® chromatograph model PROMINENCE with fluorescence detector, RF-10AXL SUPER model according to TRUCKSESS et al. (1994): an aliquot of 200 µl of the supernatant was derivatized with 700ul of trifluoroacetic acid: acetic acid: water (20:10:70, v/v/v). The chromatographic separations were carried out in a reverse phase column (silica gel, 150 x 4.6mm id., 5.0µm the size of the particles, VARIAN, Inc. Palo Alto, USA). The mobile phase used was acetonitrile, methanol and water (17:17:66 v/v/v) at a ratio of 1.5mL min<sup>-1</sup>. Fluorescence of aflatoxins derivatives was measured in excitation wavelengths and emission of λ 360nm and λ 460nm, respectively. The standard curve was constructed with different levels of AFB1, which ranged from 1.01ng ml<sup>-1</sup>, 2.02ng ml<sup>-1</sup> and 4.04ng ml<sup>-1</sup> (Sigma Aldrich® Co., St. Louis, MO USA, purity >99%). This toxin was quantified by the correlation of the heights of the peaks of the sample extract against that of the standard curve ( $y=0.0003x-0.0077$ ;  $R^2=0.99$ ). The detection limit of the analytical method was 0.4 ng g<sup>-1</sup>, based on the ration of signal-noise (3:1) and the quantification limit was set as 3 times the detection limit (1.4ng g<sup>-1</sup>).

#### Determination of aflatoxins in shrimp feed

Aflatoxin B1 (AF B1), aflatoxin B2 (AF B2), aflatoxin G1 (AF G1) and aflatoxin G2 (AF G2) were determined in shrimp feed as follows. 50g from

the samples were extracted with 150 ml methanol: water (80:20, v/v) and mixed over 60 minutes. The mixture was filtered through Whatman no.4 filter paper (Whatman, Inc., Clifton, New Jersey, USA), and an aliquot of 2.5mL was removed and 2.5mL of acetonitrile was added. The mixture was placed into a 10ml culture tube. Mycosep 228 multifunctional columns (MFC, Romer Labs®, Inc., MO., USA) were used to clean the samples. The extract was passed through the column, by a one-way valve, and through the package material. The purified extract (100µL) was collected in a column reservoir and diluted with 300µL of the mobile phase.

Detection and quantification of AF B1, B2, G1 and G2 from each sample were carried out by High Performance Liquid Chromatography (HPLC) using a SHIMADZU® chromatograph model PROMINENCE with fluorescence detector, RF-10AXL SUPER model according to the methodology proposed by TRUCKSESS et al. (1994).

The variance analysis and the SNK test were applied to compare the means and were carried using the program Sigma Stat for Windows version 2.03 (SPSS Inc.). The results were also correlated and transformed into log<sub>10</sub>.

## RESULTS AND DISCUSSION

The fungal counts of the shrimp feed were similar in farms “A” and “B” at all the cultivation phases, regardless of the collection site and package type (closed or opened), as shown in table 1. The lowest fungal count was found in farm “C” from feed from a closed package stored in phase II tank. It could be verified that the farms had acquired shrimp feed from all phases of growth with previous fungal contamination, based on the counts of the closed package samples. Therefore, feed manipulation and feed storage method used in the farms did not interfere in the amounts of isolated fungi. The samples analyzed presented values below 3.0CFU g<sup>-1</sup> (Tables 1 and 2). Fungal count was similar in the farms

Table 1 - Mean count of filamentous fungi and yeast (CFU/g) in feed supplied to shrimp at different growth stages in Piauí coastal area.

Farms	-----Phase I-----				-----Phase II-----				-----Phase III-----			
	Warehouse package		Tank package		Warehouse package		Tank package		Warehouse package		Tank package	
	Use	Closed	Use	Closed	Use	Closed	Use	Closed	Use	Closed	Use	Closed
A	2.00 <sup>a</sup>	2.10 <sup>a</sup>	1.53 <sup>a</sup>	-	1.33 <sup>a</sup>	1.60 <sup>a</sup>	1.95 <sup>a</sup>	-	1.75 <sup>a</sup>	1.62 <sup>a</sup>	1.67 <sup>a</sup>	1.46 <sup>a</sup>
B	2.66 <sup>a</sup>	2.08 <sup>a</sup>	1.41 <sup>a</sup>	1.68 <sup>a</sup>	1.69 <sup>a</sup>	1.97 <sup>a</sup>	-	1.55 <sup>a</sup>	1.91 <sup>a</sup>	1.97 <sup>a</sup>	-	1.69 <sup>a</sup>
C	-	1.64 <sup>a</sup>	2.32 <sup>a</sup>	2.16 <sup>a</sup>	2.01 <sup>a</sup>	1.67 <sup>a</sup>	1.82 <sup>a</sup>	0.65 <sup>b</sup>	2.01 <sup>a</sup>	1.49 <sup>a</sup>	1.53 <sup>a</sup>	0.75 <sup>a</sup>

ab= different letters represent different results in the same line (P<0.05%); CFU g<sup>-1</sup> = colony-forming units per gram in log<sub>10</sub>.

Table 2 - Mean count of filamentous fungi and yeast (CFU/g) in shrimp feed during the dry and rainy seasons.

Farms	-----Rainy period (AT=27° C, RH=71.5%)-----				-----Dryperiod (AT=30.9° C, RH=62.5%)-----			
	-----Warehouse package-----		-----Tank package-----		-----Warehouse package-----		-----Tank package-----	
	Opened	Closed	Opened	Closed	Opened	Closed	Opened	Closed
A	2.06 <sup>a</sup>	1.64 <sup>a</sup>	2.70 <sup>a</sup>	-	1.52 <sup>a</sup>	1.85 <sup>a</sup>	1.25 <sup>a</sup>	-
B	2.04 <sup>a</sup>	1.99 <sup>a</sup>	2.61 <sup>a</sup>	1.64 <sup>a</sup>	2.29 <sup>a</sup>	2.18 <sup>a</sup>	-	1.98 <sup>a</sup>
C	2.01 <sup>a</sup>	1.59 <sup>a</sup>	2.17 <sup>a</sup>	1.56 <sup>a</sup>	1.60 <sup>a</sup>	2.09 <sup>a</sup>	1.48 <sup>a</sup>	2.11 <sup>a</sup>

a= Equal letters in the same line and column do not present P<0.05% difference; CFU g<sup>-1</sup> = colony-forming units per gram in log<sub>10</sub>. AT=Average Temperature; RH=Relative Humidity.

(P>0.05) when the rainy and dry season collections were compared (Table 2).

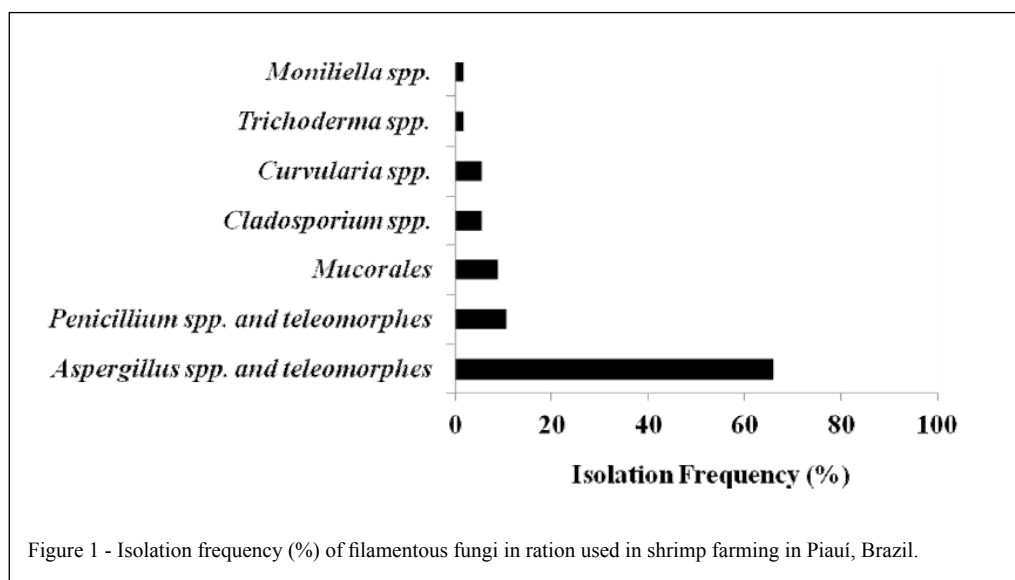
All the analyzed samples had counts under the proposed limits of 4.0log<sub>10</sub> CFU<sup>-1</sup> g<sup>-1</sup> (GMP, 2008), indicating a good microbiological quality. These results were similar to those obtained by CALVET et al. (2009), when identifying the toxigenic mycobiota in trout rations.

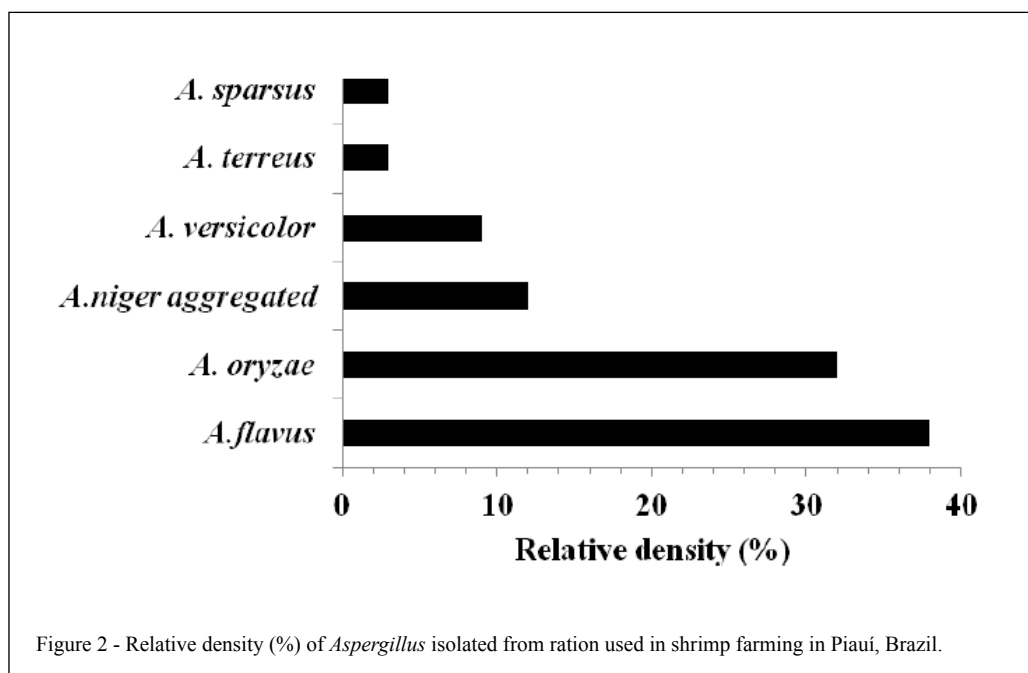
The prevalent fungi in feed used in shrimp farming in Piauí (Figure 1) belonged to the gender *Aspergillus* and its teleomorphs (66.1%). Thirty-four *Aspergillus* strains were identified; with *A. flavus* (38.2%) being the most prevalent. Other important toxigenic species, such as two strains of *A. niger* aggregated (Figure 2) were also isolated. These strains produced ochratoxin A when were qualitatively compared with standards by TLC.

Two strains of *A. flavus* produced aflatoxins B1, B2, G1 and G2. The amount of AF B1

varied from 0.39 to 0.42ng g<sup>-1</sup>; AF B<sub>2</sub> from 0.18 to 0.27ng g<sup>-1</sup>, AF G1 1.78ng/g and AF G2 0.09ng g<sup>-1</sup>. These strains can be classified as atypical *A. flavus* (TRAN-DINH et al., 1999; PILDAÍN et al., 2004; PERRONE et al., 2007). This strain isolated presents all macroscopic and microscopic morphological characteristics of *A. flavus* differentiating morphologically *A. parasiticus* (KLICH, 2002). Thus, the molecular and phylogenetic classification and identification of the strain becomes necessary, because there are no reports related to this variety of fungi in Brazil. Possibly, these strains may be a new species into the section *Flavi* not described yet.

In general, all samples showed that *Aspergillus*, the main toxicogenic fungi, were the prevalent genera. The high incidence of *A. flavus* observed in shrimp feed indicates the possible presence of aflatoxins (CAST, 2003). The isolated fungi from stored feed in closed packages could be





caused by the use of contaminated raw materials, or yet, by processing, according to BINTVIHOK et al., (2003).

From the 108 samples analyzed, only 15 (13.88%) presented contamination by aflatoxin B1 at levels that varied from 0.25ng to 360ng g<sup>-1</sup>. Two samples did not comply to the standard values recommended by the Brazilian legislation, with values above 50ng g<sup>-1</sup> for aflatoxin B1 (BRAZIL, 1988). Aflatoxin B2, G1 and G2 were not detected. The feeds used at different phases of cultivation, stored both in closed and opened packages, were found to present low fungi count in all the farms analyzed, throughout the year. Aflatoxin B1 was the only mycotoxin detected in the analyzed feed.

Intake of mycotoxins by shrimp may lead to economic losses, as these toxins interfere in their metabolism, overloading the hepatopancreas. BOONYARATPALIN et al. (2001) fed ration contaminated with aflatoxin to shrimp, confirming the presence of these toxins in their muscles after four weeks.

The low incidence of aflatoxins B1 is probably due to the high feed turnover on the farms. This practice prevents the storage of feeds for a prolonged time, inhibiting fungi growth and multiplication and, consequently, the production of mycotoxins.

## CONCLUSION

The samples of shrimp feed analyzed showed low fungal counts indicating good microbiological quality. However, aflatoxin B1 was detected in 15 samples, two out of 15 with values above the recommended by Brazilian legislation. It was noticeable the isolation of two strains classified as atypical *A. flavus* Group II (Lineage "S"), which produced aflatoxin B1 B2, G1 and G2 and were macroscopic and microscopic different of *A. parasiticus*. The molecular identification of this strains are in progress in order to differentiate from *A. parasiticus* and var *A. flavus parvisclerotigenus*.

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