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ORIGINAL ARTICLE

Fungal contamination and determination of fumonisins and aflatoxins in commercial feeds intended for ornamental birds in Rio de Janeiro, Brazil

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Significance and Impact of the Study: The mycotoxin contamination in ornamental birds is still poorly studied. There are limited numbers of reports about this subject. Most studies are still carried out in the poultry production. Due to potential contamination by fungi and mycotoxins in this feed, and the fact that there are limited data available in the world, the monitoring is highly relevant. Even if the amount of mycotoxins found is not enough to cause acute adverse effects, it is a sign that the feed will be less nutritious, and it will increase the risk of chronic mycotoxicoses. This is the first study supplying data on fungi and the occurrence of mycotoxins in Brazilian ornamental birds feed.

Keywords
aflatoxin B_1, feed, fumonisin B_1, fungi, passerines, psittacines.

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Abstract

The purposes of this study were to determine the distribution of total mycobiota, to determine the occurrence of *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. and to detect and quantify fumonisin B_1 and aflatoxin B_1 in birds' feedstuffs. Sixty samples from different commercial feeds were collected. Analysis of the total mycobiota was performed and total fungal counts were expressed as CFU g^{-1}. The isolation frequency (%) and relative density (%) of fungal genera and species were determined. Mycotoxins determination was carried out using commercial ELISA kits. The 48% of standard, 31% of premium and only 9% of super premium feed samples were found above of recommended limit (1×10^4 CFU g^{-1}). *Aspergillus* (82%), *Cladosporium* (50%) and *Penicillium* (42%) were the most frequently isolated genera. *Aspergillus niger* aggregate (35%), *Aspergillus fumigatus* (28%) and *Aspergillus flavus* (18%) had the highest relative densities. Contamination with fumonisins was detected in 95% of total samples with levels from 0·92 to 6·68 μg g^{-1}, and the aflatoxins contamination was found in 40% of total samples with levels between 1·2 and 9·02 μg kg^{-1}. Feed samples contaminated with fumonisins and aflatoxins are potentially toxic to birds.

Introduction

During the past decades, the industrialization of large countries reduced the population's contact with nature and animals, and the transition from homes to apartments was the most significant change that occurred (Rosskop and Woerpel 1996). To maintain an intimacy contact with nature, man has opted for smaller animals as pets such as fish, reptiles and birds, increasing their popularity over other ones such as the dog and the cat.

Due to the popularity of the bird, owners are becoming increasingly careful and responsible for their feeding and husbandry. It is understood that once in captivity, the nutritional needs of birds are modified extensively (Carciofi 1996). The creation of various brands of diets of birds came up with the goal of adding quality and innovation to feed these animals.

The presence of mycotoxins in animal feed, fungal secondary metabolites, is of extreme importance, because these can lead to oncogenesis, and several other pathologies.

Among the most important mycotoxins, aflatoxins produced by fungi of the genus *Aspergillus* spp. and fumonisins produced by *Fusarium* spp. are significant examples.

Aflatoxins refer to a group of mycotoxins, the most important being the aflatoxin B_1 (AFB_1) and is mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* strains (Yin *et al.* 2008). Aflatoxin B_1 is hepatotoxic and at low concentrations produces metabolic changes, leading slowly to serious health damage. The effects most frequently encountered are the decrease in growth rate and feed efficiency caused by the reduction in protein metabolism and fat absorption, suppression of immune response and hepatocarcinogenesis (Cruz 1996).

The aflatoxicosis is frequent in psittaciform and passeriform kept as pets that only receive seed diets. The most common is that the birds are simply found dead by their owners, but if exposed to low levels of toxins over a long period, they can develop chronic pathological processes (Scussel 1998).

Fumonisins (FBs), produced by *Fusarium verticillioides* and *Fusarium proliferatum,* occur worldwide and are predominantly found in maize and in maize-based animal feeds. Fumonisin B_1 (FB_1) is the most common and the most thoroughly studied, causing toxicities in animals such as equine leukoencephalomalacia and porcine pulmonary oedema, diseases long associated with the consumption of mouldy feed by horses and pigs, respectively (Voss *et al.* 2007).

Several studies about the mycotoxin contamination in animal feed by toxigenic fungi were published in Brazil in recent years (Fraga *et al.* 2007; Keller *et al.* 2007; Simas *et al.* 2007; Cavaglieri *et al.* 2009; Rosa *et al.* 2009). Nevertheless, the mycotoxin contamination in ornamental birds is still under development, and therefore, there are restricted bibliographies about this subject. Most studies are still carried out in the poultry production.

Due to potential contamination by fungi and their mycotoxins of feed given to ornamental birds, and the fact that there are limited data available in the world, the monitoring is highly relevant.

The purposes of this study were: (i) to determine the distribution of total mycobiota, (ii) to determine the occurrence of *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp., (iii) to detect and quantify aflatoxins and fumonisins in birds' feedstuffs.

Results and discussion

Mycobiota and fumonisins and aflatoxins contamination of feed intended to ornamental birds from different establishments located in Rio de Janeiro, Brazil, was studied.

Fungal total counts in dichloran rose bengal chloramphenicol agar (DRBC) ranged from $1{\cdot}0 \times 10^3$ to $2{\cdot}3 \times 10^6$ CFU g^{-1} in the three kind feed samples. Standard, premium and super premium feed had 48, 31 and 9% samples significantly over the recommended limit, respectively ($P \leq 0{\cdot}05$) (GMP 2008). The xerophilic fungi counts in DG18 ranged from $1{\cdot}0 \times 10^2$ to $2{\cdot}9 \times 10^6$ CFU g^{-1} and *Fusarium* spp. counts in SNA ranged from $1{\cdot}0 \times 10^2$–$8{\cdot}0 \times 10^3$ CFU g^{-1}.

The fungal count is an indicator of the quality of feeds. The limit to ensure the hygienic quality of products intended for animal feed is $1{\cdot}0 \times 10^4$ CFU g^{-1} (GMP 2008). In this study, a high mycological contamination was found. These results suggest a high fungal activity that could affect the palatability of feed and reduce the animal nutrients absorption and determining a low substrate quality. The percentage of samples above recommended limit was mainly in those feed samples without antifungal control. This may be due to the quality of raw materials or the presence of antifungal compounds in premium and super premium feed samples.

In this work, the used commercial feed did not detail the precise quantity of their ingredients. However, they showed different composition that allowed us to discuss the obtained results. The premium and super premium feed samples that have some antifungal control were also contaminated samples with values above the recommended limit. Food grade antioxidants (BHA and BHT) have been used safely as alternatives to fungicides to control fungal species in various food and agricultural products. These additives can limit fungal growth and the production of fumonisin and aflatoxin on natural substrates at high concentrations, however, when concentrations are suboptimal they could not inhibit fungal growth or even increase the production of toxins by these fungi (Torres *et al.* 2003; Farnochi *et al.* 2005; Passone *et al.* 2005, 2007; Barberis *et al.* 2010).

A mycological survey of the samples indicated the presence of six genera of filamentous fungi (Fig. 1). *Aspergillus* spp. was the most frequent mould of the mycobiota occurring in 96 and 88% of standard and premium feed samples, respectively, while *Cladosporium* spp. (73%) was the most frequently isolated from super premium feed samples, followed by *Penicillium* spp. in the three kinds of feeds.

Many studies have shown that most feed have species from *Aspergillus* and *Penicillium* genera as predominant flora. Our results showed that *Aspergillus*, *Cladosporium* and *Penicillium* species had the highest isolation frequencies. Rosa *et al.* (2006) and Oliveira *et al.* (2006) found similar results when studying toxigenic mycobiota of diets that were intended for broilers in four factories in Rio de Janeiro State. These authors observed that the genus *Aspergillus* was also the most frequent (41%), followed by *Penicillium* sp. (40%) and *Fusarium* sp. (15%), among

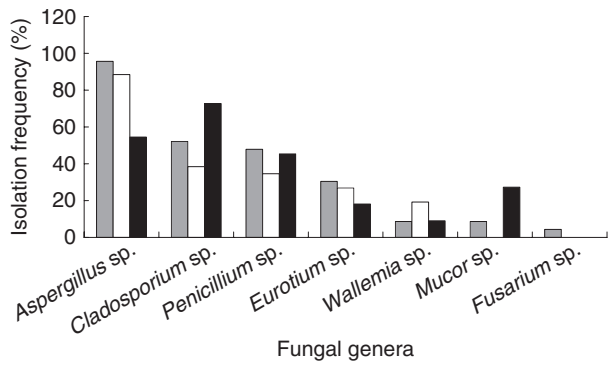

Figure 1 Frequency (%) of fungal genera isolated from feeds intended for Psittaciform and Passeriform. (▩), standard; (□), premium; (■), super premium.

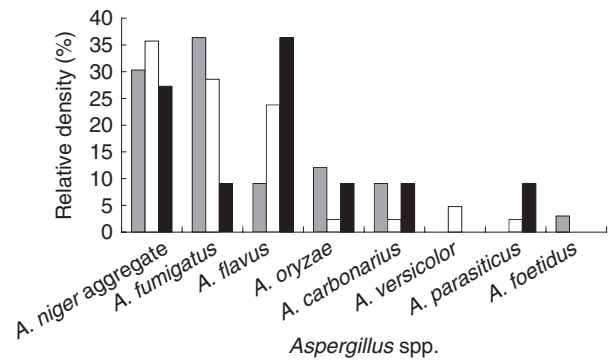

Figure 2 Relative density (%) of *Aspergillus* species in feeds for Psittaciform and Passeriform. (▩), standard; (□), premium; (■), super premium.

others. In an analysis of 60 samples of feeds from Portugal, used for ornamental birds, Martins *et al.* (2003) found that 20 among them, belonged to the Mucorales order (20%), followed by the genera *Penicillium* (15%) and *Aspergillus* (10%).

Eight species of *Aspergillus* were identified (Fig. 2). *Aspergillus fumigatus* (36%) was the most frequent from standard feed samples. *Aspergillus niger* aggregate (36%), potential OTA producer, was the most frequent from premium feed samples and *A. flavus* (36%), potential AFB_1 producer, was the most frequent from super premium feed samples.

In this study, *A. fumigatus*, *A. niger* aggregate and *A. flavus* were the most prevalent species isolated from these types of samples. A high density of *A. fumigatus* strains was found. This fungus is the main causal agent of invasive aspergillosis in humans and animals (Latge 2001) and produces several toxic metabolites. Gliotoxin, one of the major metabolites produced by *A. fumigatus*, has received particular attention because it has potent immunosuppressive, genotoxic, cytotoxic and apoptotic effects (Nieminen *et al.* 2002; Upperman *et al.* 2003). In addition, this toxin has been associated with cases of clinical aspergillosis in turkeys (Richard *et al.* 1996). Martins *et al.* (2003) analysed diets for pets, including ornamental birds, found similar results, where *A. flavus* was present in 23% of the samples. Fraga *et al.* (2007) and Rosa *et al.* (2006) analysed samples of corn and feeds for broilers in the State of Rio de Janeiro. *Aspergillus flavus*, *Aspergillus candidus* and *Eurotium* species were more observed. They also found *A. niger* aggregate, *Aspergillus sydowii* and *Aspergillus versicolour*. Note that every cited species was isolated in different percentages in our work, with the exception of *A. sydowii* and *A. candidus*. The presence of these strains in ornamental birds' nutrition implies a high risk of mycotoxicosis.

As for the genus *Penicillium*, four species were identified in this study. *Penicillium citrinum* was the most frequently isolated specie (70, 90 and 100%) from standard, premium and super premium feed samples, respectively. Other species as *P. citreonigrum* (5% in the three kind of feeds), *P. brevicompactum* (10%; only isolated from Standard feed) and *P. islandicum* (4% from the three kind of feeds) were isolated. The only species of *Fusarium* isolated was *F. verticillioides* (100%) in standard feed samples. *Penicillium citrinum* was the species most frequent from the three kind feed. By analysing feeds for broilers, pigs and horses from Spain, Bragulat *et al.* (2008) also found, with different percentages, the *Penicillium* species isolated in our work (*P. citreonigrum, P. brevicompactum, P. islandicum)*, with the exception of *P. citreonigrum* which was not isolated by the authors. These species produce different toxic fungal metabolites as citrinin and citreoviridin (Pitt and Hocking 1997). There is little information regarding the toxicological effects of these mycotoxins in animals (CAST 2003).

In this study, *F. verticillioides* was the only species of *Fusarium* found. Campos *et al.* (2008) and Juri *et al.* (2009) analysed feeds used in pets diet also found similar result. Corn is usually the main ingredient of diets intended for horses, as well as birds. *F. verticillioides, F. proliferatum* and *F. subglutinans* are the species most commonly associated with contamination of corn and subproducts (Mallmann and Dilkin 2007).

The present study has shown the occurrence of two groups of mycotoxins, fumonisins and aflatoxins in feed intended for ornamental birds' consumption. Contamination with fumonisins was detected in 95% of total samples with levels from 0·92 to 6·68 $\mu g\ g^{-1}$, and the aflatoxins contamination was found in 40% of total samples with levels between 1·2 and 9·02 $\mu g\ kg^{-1}$ (Table 1). Scudamore *et al.* (1997) found OTA contamination in one of 15 wild bird feed samples studied. In

Table 1 Fumonisin B_1 and aflatoxin B_1 levels in ornamental birds feed samples

		Bird feed samples		
	Mycotoxins	Standard	Premium	Super premium
FB_1	Media ± SD (μg g^{-1})*	2·67 ± 1·64[a]	3·10 ± 1·30[a]	2·10 ± 0·84[a]
	Minimum and maximum values (μg g^{-1})	1·02–6·68	0·92–5·36	1·39–3·08
	Frequency (%)†	91	100	91
	Samples over limits (%)‡	8·7	7·7	0
AFB_1	Media ± SD (μg kg^{-1})*	1·73 ± 2·16[a]	1·76 ± 2·65[a]	1·33 ± 2·69[a]
	Minimum and maximum values (μg kg^{-1})	1·20–6·88	1·30–7·42	1·73–9·02
	Frequency (%)†	48	35	36
	Samples over limits (%)‡	0	0	0

*SD: standard deviation.

†Contamination frequency (%): percentage of samples contaminated with mycotoxin.

‡Percentage of samples contaminated with levels over the recommended: AFB_1: 20 μg kg^{-1}, FB_1: 5 μg g^{-1} (GMP 2008). Letters in common are not significantly different according to Fisher's protected LSD test ($P < 0·05$).

our study, silicates or esterified glucomannans were part of the composition of super premium feeds and this fact could explain the lower mycotoxin level found in this kind of substrate. Positive samples were not confirmed by HPLC.

Our results indicated that only 7% of the samples had levels of fumonisins above those permitted by the European commission, which is 5 μg g^{-1} for animal feed (EU 2006), and that 100% of the samples were below the maximum limit of aflatoxins allowed by Brazilian law, that is up to 50 μg kg^{-1} (MAPA 1988). This also occurred when comparing with the limits accepted by the European Union, which is 20 μg kg^{-1} (EU 2003). However, the effects on the health of ornamental birds, as well as the economic losses with the cost of medicines and veterinary assistance, generate the need for new laws and control mechanisms more effective.

A co-occurrence of fumonisins and aflatoxins was present in 43% standard, 35% premium and 27% super premium analysed feed samples. The co-occurrence of AFB_1 and FB_1 is a topic for further investigation, as there is no description of the effects of this interaction in psittaciforms and passeriforms. This is alarming because both toxins interact, accelerating the formation of liver cancer in experimental animals (Gelderblom *et al.* 2002). In studies conducted in chickens by Kubena *et al.* (1995) an additive effect of these mycotoxins was shown.

The regulation of mycotoxins in animal feed has the main focus in the livestock production, with less attention to the pets. European standards and Brazilian law are actually intended for agricultural species and probably they may not be applicable to ornamental species as studies on long-lived bird species (i.e. psittacines compared with poultry) indicate that some of them such as budgerigars have cellular resistance to oxidative damage so mycotoxin levels may be different to those accepted for poultry. Until present, it does not exist a specific legislation on mycotoxins for ornamental birds.

However, the presence of mycotoxins indicates the existence of contamination. As raw materials are the main responsible of the moisture level and fungal contamination found in feed, the first important step in controlling moisture in feed is to control it in the raw materials from which the feed is prepared.

This is the first study supplying data on fungi and the occurrence of mycotoxins in Brazilian ornamental birds feed.

Materials and methods

Sampling

Sixty (60) samples in total from different commercial feeds were randomly collected from Brazilian industries located in Rio de Janeiro, Brazil, from March to December 2009.

The classification of samples was based on fungal control used by the manufacturer (the presence of antioxidants, fungistatic's additives and adsorbents of mycotoxins) being the samples classified into three groups: *Standard* feeds (23 samples without control), *Premium* feeds (26 samples with at least one type of control) and *Super Premium* feeds (11 samples with more than two types of control). All these samples were within the expiry date and correct conditions of storage.

Birds' feed composition

Standard feeds were composed by corn, soy, bread crumbs, vegetable oil, vitamin premix and sodium chloride. The *Premium* feeds by corn, soy, bread crumbs, vegetable oil, vitamin premix, sodium chloride, sunflower seeds, yeasts, eggs, fungal control (hidroxibutilanisol – BHA or

hidroxibutiltolueno – BHT). *Super Premium* feeds by corn, soy, wheat, broken rice, yeasts, eggs, citrus pulp, DL methionine or L-lysine, vitamin premix, sodium chloride, fungal control (BHA or BHT, propionate acid) and silicates or glucomannan esterified.

Mycological isolation and identification

Analysis of the mycobiota was made by the plate dilution spread method onto DRBC, a general medium used for estimating total mycobiota, dichloran glycerol 18% agar (DG18) (Pitt and Hocking 1997) a low a_W medium that favours xerophilic fungi development and Nash–Snyder (SNA) culture media that allows *Fusarium* spp. development (Nelson *et al.* 1983). Total fungal counts were expressed as CFU g^{-1}. 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).

The identification in the genus level was performed according to Samson *et al.* (2000). The fungal colonies identified as *Aspergillus* and *Penicillium* were maintained in malt extract agar (MEA) and the *Fusarium* sp. ones on V-8 *juice* agar (V8) for later identification of species. The strains of fungi belonging to these genera were then identified according to taxonomic keys appropriate for each particular group (Nelson *et al.* 1983; Pitt 1988; Klich 2002). After mycological analysis, each subsample was stored at 4°C until mycotoxin analyses.

Mycotoxins determinations

For detection and quantification of aflatoxins and fumonisins, commercial ELISA kits developed by Romer Labs Singapore Pte. Ltd. were used (AgraQuant® Total Aflatoxin Assay and AgraQuant® Total Fumonisin Assay ELISA strip reader (Drake® model Quick ELISA, São Paulo, Brazil). The standards in aflatoxins kits were: 0, 1·0, 2·0, 4·0, 10·0 and 20·0 $\mu g\ l^{-1}$ (ppb), and in fumonisins were: 0, 0·25, 1·0, 2·5 and 5·0 $\mu g\ ml^{-1}$ (ppm). The manufacturer's instructions were strictly followed. The experiments were conducted in duplicate. The method for aflatoxins and fumonisins determination by ELISA was in house validated. For the method validation, standard solutions and spiked samples were employed. For the ELISA test, the detection limits for aflatoxins and fumonisins were 1·0 $\mu g\ l^{-1}$ (ppb) and 0·25 $\mu g\ ml^{-1}$ (ppm), respectively, and the analyte recovery was 102 and 97·1%, respectively. The linearity of the calibration curve was verified by determining the coefficient of determination (R^2) and was up to 0·98 for both mycotoxins. The precision (repeatability) and intermediary precision (intralaboratory reproducibility) for the aflatoxins were 12 and 17%, respectively, and for fumonisins, the values were 16 and 21%, respectively. The performance of the methods exhibited good accuracy (z-score <2).

Statistical analysis

The data were subjected to analysis of variance (ANOVA). Before ANOVA, all data were transformed using a logarithmic function log10 (x + 1). Duncan's test was used to compare the data of the various fungal culture media, and also to compare the measurement data of mycotoxins. Analyses were performed using the software PROC GLM in SAS (SAS Institute, Cary, NC, USA).

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