

Fungal flora and mycotoxins detection in commercial pet food

Flora fúngica e pesquisa de micotoxinas em alimentos para animais de companhia

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Summary: The role of fungi and mycotoxins contamination in pet food is not conveniently evaluated in Portugal. The authors report the identification of mycoflora and mycotoxins determination in 60 samples of dry pet food; 20 for dogs, 20 for cats and 20 for domestic birds (10 for canaries and 10 for parrots) acquired in different pet shops. Moulds evaluation was determined using a conventional method. The mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), fumonisin B1 (FB1) and deoxynivalenol (DON) were analysed by high performance liquid chromatography (HPLC). The detection limits were: 1.0 µg/kg for aflatoxins, 2.0 µg/kg for ochratoxin A, 10 µg/kg for fumonisin B1 and 100.0 µg/kg for deoxynivalenol. All samples had very low fungi counts. The predominant genera were *Aspergillus* (58.3%), *Penicillium* (38.3%) and *Mucor* (38.3%). Aflatoxins were not detected in any sample. Ochratoxin A was detected in five samples (8.3%) with levels ranging from 2.0 to 3.6 µg/kg. Fumonisin B1 and deoxynivalenol were present in three samples (5%) with levels ranging from 12.0 to 24.0 µg/kg and 100 to 130 µg/kg respectively. The mycotoxins (OTA, FB1 and DON) were detected only in dog food samples. The data presented in this study confirm the fungi contamination and the presence of the mycotoxins in foods for pets that can possibly represent an hazard for these animals.

Key words: fungi, mycotoxins, HPLC, pet food

Resumo: O papel das contaminações fúngicas e da presença de micotoxinas nos alimentos destinados a animais de companhia não está devidamente avaliado em Portugal. Os autores referem a identificação da micoflora e a presença de micotoxinas em 60 amostras de alimentos secos para animais de companhia de diferentes marcas adquiridas em lojas da especialidade, sendo 20 amostras de alimentos para cães, 20 para gatos, 10 para periquitos e 10 para catatuas. A contagem e identificação dos agentes fúngicos foram efectuados por métodos micológicos convencionais e a pesquisa de micotoxinas, aflatoxinas (AFBs), ocratoxina A (OTA), fumonisin B1 (FB1) e deoxynivalenol (DON) foi efectuada por cromatografia líquida de alta resolução (HPLC). Os limites de detecção foram: 1,0 µg/kg para aflatoxinas, 2,0 µg/kg para ocratoxina A, 10 µg/kg para fumonisin B1 e 100 µg/kg para deoxynivalenol. O nível de contaminação fúngica foi muito baixa em todas as amostras. O género mais frequente foi *Aspergillus* com 58,3 %, seguido de *Penicillium* e de *Mucor* ambas em 38,3% das amostras positivas. Nenhuma amostra continha aflatoxinas. Ocratoxina A foi detectada em 5 amostras (8,3%)

com níveis que oscilaram de 2,0 a 3,6 µg/kg. Fumonisin B1 e deoxynivalenol estavam presentes em três amostras (5,0%) com níveis de contaminação de 12,0 a 24,0 µg/kg e 100,0 a 130,0 µg/kg respectivamente. Estas micotoxinas (OTA, FB1 e DON), foram detectadas apenas em alimentos para cães. Estes resultados confirmam a presença de flora fúngica e de micotoxinas em alimentos para animais de companhia, representando um perigo sanitário potencial.

Palavras chave: fungos, micotoxinas, HPLC, animais de companhia

Introduction

Information about fungi associated with food and feeds is important in assessing risk of mycotoxin contamination. Dog and cat food are prepared with vegetables and/or meat (chicken, beef, turkey and fish), cereal grains (maize, sorghum and rice), fat, vitamins and minerals. During its manufacturing, food can be contaminated with mould spores, especially when cereal grains are ground and food is granulated (Suárez, 1999). Food marketed for birds contain a wide range of nuts and cereal grains.

The filamentous moulds most commonly found in stored cereal grains are *Aspergillus*, *Penicillium* and *Fusarium* species; they are ubiquitous, can cause food spoilage and biodeterioration, and are capable of producing many different mycotoxins. *Aspergillus* species (*A. flavus*, *A. parasiticus*, *A. nomius*, *A. fumigatus*, *A. versicolor* and *A. ochraceus*) are some of the more common toxigenic species (Betina, 1989). *A. flavus*, *A. parasiticus* and *A. nomius* produce the most carcinogenic mycotoxins, the aflatoxins (Kurtzman *et al.*, 1987), whereas as many as 10 species of *Aspergillus* produce ochratoxin A (Abarca *et al.*, 1994, Téren *et al.*, 1996, Joosten *et al.*, 2001, Martins *et al.*, 2002). Several *Penicillium* are commonly involved in food spoilage, and most of them produce more than 10 different toxic fungal metabolites (cyclopiazonic acid, patu-

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lin, citrinin, penicillic acid) (Frisvad and Filtenborg, 1989; Martins and Martins, 1999). Although many *Fusarium* species exist in nature, only a small number infect cereal crops in the field and produce mycotoxins (fumonisins, zearalenone and trichothecenes) (Marasas, 1992). Mycotoxin production is favoured by high humidity and high water activity and has been detected in many agricultural crops.

Dogs are particularly sensitive to the acute hepatotoxic effects of aflatoxin (Newberne *et al.*, 1955; Newberne *et al.*, 1966; Scudamore *et al.*, 1997). Aflatoxin B₁ (AFB₁) is classified into the difurocoumarocyclopentenone series and the difurocoumarolactones. AFB₁ is metabolically converted to a variety of stable metabolites (aflatoxicol, aflatoxin M₁ and aflatoxin Q₁) *in vivo* or *in vitro* in the presence of NADPH-generating system and cytochrome P-450, which is a requisite step in the formation of the putative active biological effects: carcinogenicity, DNA binding, cytotoxicity and bacterial mutagenicity (Coulombe *et al.*, 1984).

The most important producers of ochratoxins are *Penicillium verrucosum* in temperate climates, *P. viridicatum*, and *Aspergillus ochraceus* in warmer and tropical parts of the world. Ochratoxin A (OTA) can occur naturally in cereal and cereal-based foods (Krogh, 1987). OTA is the most common and consists of a dihydrocoumarin moiety linked to an L-phenylalanine functional group (Kuiper-Goodman and Scott, 1989). OTA is a potent nephrotoxic and nephrocarcinogenic mycotoxin. The morphological renal lesions are characterized by degeneration of proximal and distal tubules and interstitial fibrosis.

Fumonisin is a toxic compound first isolated and characterized in 1988 (Gelderblom *et al.*, 1988). These mycotoxins are mainly produced by *Fusarium moniliforme* strains which occurs on cereals and especially as a major fungal contaminant on maize. Fumonisin B₁ and B₂ (FB₁ and FB₂) are diesters of tricarballylic acid and polyhydric alcohols and as they have similar structure to sphingosine they can interfere with sphingosine metabolism, blocking the biosynthesis of complex sphingolipids and ceramides. FB₁ has been pointed as a natural cause of leukoencephalomalacia in horses, pulmonary edema in porcine and oesophagi cancer in humans (Scott, 1993).

Deoxynivalenol (DON), also known as vomitoxin, is one of a group of closely related secondary fungal metabolites, the trichothecenes. DON is one of the toxic 12,13-epoxytrichothecenes produced by various species of *Fusarium*, particularly *F. graminearum*. Wheat, corn and barley are particularly affected and are known to cause feed refusal, emesis and growth depression in swine and chickens. DON is inhibitor of protein and DNA synthesis and also affects food intake, body weight and suppresses humoral and cellular immune function (Peslka and Bondy, 1990).

Mycotoxins are increasingly being shown to occur in the conidia of toxigenic fungi. The presence of single

or multiple mycotoxins in airborne conidia could represent a potential health risk to both animals and man (Lewis *et al.*, 1994)

The purpose of this study was to give information about the fungal contamination and levels of mycotoxins in pet foods marketed in Lisbon, Portugal.

Material and methods

Samples

A survey was carried out to evaluate the fungal contamination and the possible presence of aflatoxins, ochratoxin A, fumonisins B₁ and B₂ and deoxynivalenol in 60 samples of dry pet food; 20 for dogs, 20 for cats and 20 for ornamental birds (10 for canaries and 10 for parrots) corresponding to different imported brands. Samples were acquired in pet shops.

Mycological determination

Ten grams of each sample were homogenized for 3 min in 90 ml (10⁻¹ suspension) peptone water (Oxoid, code CM 9, Basingstoke, England) in a Colworth 400 Stomacher (Seward Medical, London, UK). Ten-fold dilutions were prepared till 10⁻⁴.

For enumeration and identification at genus level of moulds and yeast, 1 mL of each dilution was spread into each of five plates (0.2 mL/plate) of Cook rose bengal agar with chloranphenicol (DRCB, Oxoid, CM 727, Supplement - chloranphenicol SR 78) (King *et al.*, 1984) and incubated at 28 °C for 5 days. Each isolated mould colony was observed microscopically for morphological characterization and identification (Domsch *et al.*, 1980; Samson and Pitt, 1989).

Mycotoxins determination and quantification by HPLC

Aflatoxins

The samples were analysed for the quantification of AFs using immunoaffinity columns (Aflaprep, Code PO7) supplied from Rhône-diagnostics Technologies Ltd (Spain), and quantified by high pressure liquid chromatography (HPLC) according to the method described by Stroka *et al.* (2000). The detection limit is 1 µg/kg. The AFs were extracted from 50 g of sample with a solvent mixture of methanol/water (8+2,v/v). The sample extract was filtered, diluted and applied to an immunoaffinity column containing antibodies specific to aflatoxins B₁, B₂, G₁ and G₂. The aflatoxins were eluted with methanol. Standard AFs B₁, B₂, G₁ and G₂ were purchased from Sigma-Aldrich (Ref. A-6636, A-9887, A-0138 and A-0263 respectively) (Quimica S.A. Spain).

Ochratoxin A

OTA determination was performed according to the method described by Entwistle *et al.* (1999). The

detection limit is 2 µg/kg. OTA was extracted from the 25 g of the sample with acetonitrile. The extract was cleaned up by passing through an immunoaffinity column (OchraPrep, Code P 14B, Rhône-Diagnostics Technologies Ltd, Spain), and the OTA was eluted with methanol/water/acetic acid and separated by reverse phase HPLC. Standard of OTA was purchased from Sigma (Ref.O-1877).

Fumonisin

Extraction, purification and HPLC quantification of FB1 was performed by the technique described by Shephard *et al.* (1990). The detection limit is 20 µg/kg. Briefly the technique may be described as following: 25 g of sample were blended with methanol/water and filtered. An aliquot of the filtered was applied to a Bond-Elut strong anion-exchange (SAX) cartridge 3 cc (Ref: Wat 020850) previously equilibrated with methanol/water and washed with methanol/water followed by methanol and the toxins were eluted with an acetic acid/methanol solution. The eluate was evaporated to dryness and the dried sample was redissolved in methanol, and an aliquot was derivatized with o-phthalaldehyde (OPA) (Sigma-P-0657) and 2-mercapto-ethanol (Sigma M-6250) prior to separation on a reversed phase HPLC system. Standard of FB1 was purchased from Sigma (Ref. F-1147)

Deoxynivalenol

DON analysis was carried out following the method described by Cahill *et al.* (1999). The limit of detection was 100 mg/kg. A sample of 50 g was extracted in distilled water by blending, filtered through both a fluted and glass microfiber filter paper and applied to an immunoaffinity column (DONtest-VICAM). Subsequently the column was washed with distilled water and the toxin was eluted from the column with methanol, evaporated to dryness in a rotary evaporator and re-dissolved in 300 µl of acetonitrile-water. DON was obtained from Sigma (Ref. D- 0156).

Results

All positive samples showed low levels of contamination (10¹ to 10² cfu/g) (Table 1). The most frequent mould occurring in dry pet food was *Aspergillus* (58.3%) followed by *Penicillium* spp. and *Mucor racemosus*, which occurred each in 38.3 % of the samples. Among the *Aspergillus*, the percentage *Aspergillus niger* was high (55.0 % in dog food, 40.0 % in cat and 10.0 % in bird food). In general dog food had higher percentage of contamination than the other foods. The samples of bird seeds had lower contamination. Yeasts were not present in any sample.

The mycotoxins results in pet food are shown in Table 2. Eighty-two percent of the samples contained no measurable concentrations of mycotoxins. The presence of mycotoxins was observed only in dog food. No aflatoxins were detected. Ochratoxin A was detected in five samples (8.3 %) with levels ranging

Table 1 - Frequency and level of mycoflora isolated in pet foods

	Dog Food samples (% positive)	Cat Food samples (% positive)	Bird Food samples (% positive)	% total samples
Fungi	10 ¹ - 10 ² cfu	10 ¹ - 10 ² cfu	10 ¹ - 10 ² cfu	
<i>Aspergillus flavus</i>	8 (40.0)	6 (30.0)	0 (0.0)	58.33
<i>Aspergillus niger</i>	11 (55.0)	8 (40.0)	2 (10.0)	
<i>Penicillium</i> spp.	13 (65.0)	7 (35.0)	3 (15.0)	38.33
<i>Mucor racemosus</i>	11 (55.0)	8 (40.0)	4 (20.0)	38.33

from 2.0 to 3.6 µg/kg, with average concentration of 2.8 µg/kg. Fumonisin B1 was present in three samples (5%) with levels ranging from 12.0 to 24.0 µg/kg, with average concentration of 17.3 µg/kg and deoxynivalenol was also present in other three samples, with levels ranging from 100 to 130 µg/kg, with average concentration of 116 µg/kg (Table 2).

Table 2 - Frequence of mycotoxins in dog food

Mycotoxins	Positive samples (%)	Min - max (µg/kg)	Average (µg/kg)
Aflatoxins	0 (0.0)	-	-
Ochratoxin A	5 (25.0)	2.0-3.6	2.8
Fumonisin B1	3 (15.0)	12.0-24.0	17.3
Deoxynivalenol	3 (15.0)	100.0-130.0	116.0

Discussion

The results indicate that there is little evidence of significant mycotoxins contamination of pet food. Few samples were contaminated with OTA, FB1 and DON, and its levels were low with no toxicological concern.

Cereals in pet food are good matrix for fungi growth (González *et al.*, 1997). Abarca *et al.* (1994) found moulds of genera *Penicillium*, *Aspergillus* and *Fusarium* in mixed feed for cattle, pig and poultry. Dalcero *et al.* (1998), got similar results in poultry feed. In 1997, Scudamore *et al.* (1997), found in pet food for domestic pets and wild birds, *Penicillium* and *Eurotium*. Bueno *et al.* (2001) in 21 samples of pet food found *Aspergillus* (62 %), *Rhizopus* (48 %) and *Mucor* (38 %). In Portugal, surveys were carried out concerning the presence of mycoflora and mycotoxins in component raw materials and feedstuffs (Martins and Martins, 2000; Martins and Martins, 2001 a), 2001 b), 2001 c); Martins *et al.*, 2002 b). These researchers identified *Penicillium*, *Aspergillus*, *Fusarium*, *Mucor* and yeasts.

The presence of toxigenic fungi does not indicate mycotoxin production. Toxins may persist long after vegetative growth has occurred even after moulds death. Therefore, the presence of certain fungi implicates a potential risk for animal health (Bueno *et al.*, 2001).

There is few information concerning surveys of mycotoxins in pet food. Scudamore *et al.* (1997), in

100 samples of pet food (35 samples of cereal-based dog and cat food, 15 samples of domestic bird seeds and 15 samples of wild bird food) examined for aflatoxins, ochratoxin and fumonisin (only in 20 samples) found aflatoxin B1 in low level in a sample of cat food, and a concentration of 370 µg/kg in one sample of peanuts marketed for wild birds. OTA was detected in 10% of samples but in low concentrations, the highest of 7 µg/kg occurring in a sample of bird food. Fumonisin were found in 30% of the 20 samples tested with a maximum of 750 µg/kg total fumonisins being found in a sample of cat food. Henke *et al.* (2001) in a study on a total of 142 wild bird seed, in Texas reported the presence of aflatoxin at level of non-detectable to 2.8 µg/kg.

In Portugal and in other UE Member States, maximum levels of aflatoxins and OTA contamination are stated by official regulations (Regulation/CE n° 466/2001 of 8 March; Dir. 2002/26/EC of 13 March). According to FAO, (1997) several countries established maximum levels of wide range of mycotoxins, including DON, in foodstuffs, dairy products and animal feedstuffs.

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