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Mycobiota and Concentration of Ochratoxin A in Concentrated Poultry Feed from Venezuela

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Abstract: The main objective of this study was to evaluate the frequency distribution of mycobiota and the concentration of Ochratoxin A (OTA) in 50 samples from one company of commercial brand poultry feed produced in Venezuela. The concentration of OTA in the samples analyzed was determined using the competitive ELISA method. The most frequently isolated genera of moulds were *Aspergillus* (36%) and *Penicillium* (20%). Of these genera, the most frequently isolated species were *Aspergillus flavus*, *Aspergillus terreus* and *Penicillium citrinum*. Ochratoxigenic species such as *Eurotium herbariorum*, *Aspergillus niger*, *Aspergillus ochraceus* and *Aspergillus glaucus*, were also found with lower frequency. *Rhodotorula mucilaginosa* was the only yeast isolated. 94% of the samples presented contamination by OTA in a range between 2.558 and 31.978 μg kg^{-1} feed and 42% of them presented OTA levels from 10 up to 20 μg kg^{-1}. The findings of this investigation show that 84% of the samples of concentrated feed for meat poultry surpass the maximum permitted limit for OTA of 5 μg kg^{-1}, established in the majority of countries in which regulations are placed.

Key words: Mycobiota, ochratoxin A, poultry feed

INTRODUCTION

Ochratoxins are defined as secondary metabolites produced by moulds that contaminate a large variety of food products such as cereals, pulses, nuts, coffee, beer, wine, meat and cheese and they are the cause of major problems of nephrotoxicity, carcinogenicity, teratogenicity and immunotoxicity in animals and humans. Ochratoxin A (OTA) is the main toxin within this group (Miller, 1995; Murphy *et al*., 2006; López *et al*., 2007).

Several investigations have shown that the most common species producing ochratoxins are: *Aspergillus ochraceus*, *A. niger*, *A. glaucus*, *A. carbonarius*, *A. alliaceus*, *Eurotium herbariorum* and *Penicillium verrucosum* (López *et al*., 2007; Magnoli *et al*., 2007).

It has also been proved that *P. verrucosum* is the dominant productive species of Ochratoxin A in food products for humans and animals in regions of low temperature, such as the north of Europe and Canada (Larsen *et al*., 2001). Similarly, species such as *A. niger*, *A. flavus* and *A. ochraceus* are important sources of OTA in South America's countries such as Argentina, Ecuador and Brazil (Rosa *et al*., 2006; Magnoli *et al*., 2007).

The European Food Safety Agency (EFSA) has recommended that the levels of OTA for humans should be reduced below 5 ng kg^{-1} of body weight per day. On the other hand, in Canada a tolerable ingestion is estimated to be between 1.5 and 5.7 ng kg^{-1} of body weight per day and the Join FAO/WHO Expert Committee on Food Additives (JEFCA) permit an maximum ingestion of 100 ng kg^{-1} of body weight per week, which corresponds to approximately 14 ng daily uptake of OTA per kg of body weight (Walker, 2002).

The most affected animals by the consumption of food products contaminated with OTA tend to be cattle, goats, sheep, pigs and at an especially high level, fowl. It has therefore been determined that ochratoxicosis in meat poultry causes weight loss, growth problems and death. Embryonic mortality in chickens caused by the teratogenic effects of OTA has also been observed. Immuno-suppression in these birds is mainly derived from thymic atrophy. This mycotoxin accumulates mainly in the kidneys and in lower concentrations in the liver and thighs of the birds and it is distributed in the yolk and the albumin of the eggs. These effects are observed mainly due to the fact that the OTA is produced very rapidly in poultry feed in conditions of elevated humidity and temperature. In particular, the raw materials for producing this kind of feed are for the most part cereals (corn, wheat, sorghum and rice) and by-products of cereals, which are the principal source of energy in concentrated feeds and constitute over 50% of the total ingredients in rations for

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meat poultry. However, they also constitute the main substrate for the growth of fungi and consequently, for the formation of mycotoxins, thus compromising the health of these birds and of the humans who will later consume them, as part of the food chain (Vilariño *et al*., 1996; Vivas, 2005; López *et al*., 2007).

Fungal contamination of feed concentrates for birds may occur as a result of external or internal origins. Externally, it occurs when the raw materials that have been contaminated during sowing, reaping or storing are used, while internally it occurs when the composite feed is processed, whether this is during its storage in silos, or because of contamination during transportation, elevation, mixing or in the interior of pipes-especially in the bends and joints-which is generally a result of a lack of hygiene in the installations (Saunders *et al*., 2001; Scudamore, 2005).

Bacon *et al*. (1973) were the first who described that *A. ochraceus* was capable of producing OTA and that temperatures of approximately 30°C and high levels of humidity favored the production of this mycotoxin in whole foods for farmyard birds. The raw materials used in livestock feed, especially cereals and their by-products, constitute an ideal substrate for the growth of toxigenic fungi and for the production of mycotoxins, among which OTA needs to be highlighted. Micotoxin contaminated feed affect agricultural production, since it generates large economic losses associated with the animals performance parameters and illness and death of the animals. Additionally, it affects consumers who may consume contaminated food down in the food chain (Benneth and Klich, 2003).

Miller (1995) stated that farmyard birds are mainly affected by the reduction in the index of growth and the reduction in egg production. Rosa *et al*. (2006) revealed that the presence of OTA in poultry feed affects the industry since the animals suffer from problems associated with growth and it makes them more susceptible to subclinical intoxications. López *et al*. (2007) has also indicated that ochratoxicosis affects the carbohydrate metabolism, generating an accumulation of glycogen in the liver. Ochratoxicosis in meat poultry causes lesions in the kidneys and liver leading to weight loss and reduced growth. They also indicated that it produces embryonic mortality, immuno-suppression and death. OTA is mainly distributed in the kidneys and can pass to the yoke and albumin of eggs. López *et al*. (2007) indicated that LD_{50} in poultry through the oral administration of OTA is 3.3 mg kg^{-1} of body weight, which means a high risk of mortality for these birds.

Human exposure to OTA comes mainly from the daily intake of cereal grains. However, in some parts of Europe it has been detected that exposure originates from the consumption of animal products, especially pork and products based on pig blood (Miller, 1995). Walker (2002) has presented data indicating that cereals, wine, grape juice, coffee and pork are the main sources of human exposure to OTA, at levels of 58, 21, 7, 5 and 3% of total ingestion, respectively. The detection of the presence of OTA in Europe in pork products sold in retail establishments has shown that this toxin may pass from animal feed to animal-based products (Murphy *et al*., 2006). This suggests that the same is also possibly happening in meat poultry, destined principally for human consumption.

Being poultry one of the main components of human diet and being concentrated feeds one of the most susceptible products to be contaminated by OTA, it is necessary to evaluate the mycobiota present in these feeds in order to determine the presence of toxigenic fungi and OTA.

MATERIALS AND METHODS

This study was carried out at the end of 2007 and beginning of 2008 in the Departamento de Technología de los Alimentos at the Universidad de Oriente in Venezuela, with the collaboration of the Departamento de Sanitat id' Anatomia Animals at the Universitat Autònoma de Barcelona (Spain).

Isolation and identification of moulds yeasts and yeast: Fifty bags of 35 kg each were selected, corresponding to 10% of a particular batch of a small commercial brand of poultry feed. The ingredients used for the samples of concentrated poultry feed in this research were mainly cereals (50%), oilseed and mill by-products, as well as animal-based protein, synthetic amino acids, macro and micro minerals, vitamins, antioxidants, animal fats and vegetable fats. They were stored in a poultry feed distributor in the state of Sucre, applying the random sampling method specified by the International Commission on Microbiological Specifications for foods. One representative sample (of approximately 100 g) was taken from the surface, center and bottom of each bag selected. The average temperature and the relative humidity of the feed ingredients store were 29.1°C and 64.4% (range: 63-70%), respectively. The samples were transferred to the laboratory in sterile, previously identified plastic bags and they were processed immediately.

Ten grams of each sample were weighed out on scales and placed aseptically and individually in 250 mL sterile flasks containing 90 mL of physiological sterile

saline solution. The flasks were kept in constant mechanical agitation for 10 min. Ten fold dilutions were performed until 10^{-5} was reached. For this, 1 mL of each of the prepared dilutions was taken and mixed with 15 mL of Malt Extract Agar (MEA) to 2%, previously melted and maintained at 45°C, to which 30 μg mL^{-1} of tetracycline chloride was added to prevent bacterial growth. The plates were seeded in duplicate and incubated at 28±2°C for 5 days. All the colonies with different macroscopic characteristics were chosen and were isolated separately in test tubes containing MEA and they were incubated at 28±2°C for 5 days.

The identification of moulds was carried out by a microscopic and macroscopic study of the isolated colonies, using conventional mycology techniques (Samson, 2000). Yeast-like appearance colonies were also studied by means of their macroscopic and microscopic characteristics. The identification was performed using the API 20C AUX (Biomérieux).

Determination of ochratoxin A: The presence of OTA was determined using the ELISA method (Biopharm, Germany). Briefly, the extraction of OTA was as follows: In a flask, 5 g of the sample, previously ground, was mixed with 12.5 mL of 70% methanol and then shaken vigorously for 3 min. The mixture was filtered using Whatman No. 1 filter paper and 1 mL of the filtered liquid was diluted with 1 mL of distilled water. The absorbency was measured at 450 nm using an ELISA reader (Biotek ELX800). The results obtained were expressed in ppb (μg kg^{-1}).

Statistical analysis: The results obtained were analyzed by percentage analyses method (%) and the Chi-square test (χ^2) with a significant level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the frequency of isolated mould genera in the poultry feed samples, where a highly significant difference was obtained ($\chi^2 = 17,1668$; $p<0.05$), being the genus *Aspergillus* and *Penicillium* the most frequently isolated. These results coincided with those published by Labuda and Tančinová (2006), Oliveira *et al.* (2006) and Rosa *et al.* (2006), who found that the *Aspergillus* and *Penicillium* were the most frequently isolated genera in the feed samples analyzed for farmyard birds. Also, the earlier results showed that the genus *Aspergillus* is predominant in tropical environments, as is the case in Venezuela (Zimmerli and Dick, 1996; Pitt and Hocking, 1997).

Table 2 shows the frequency of fungal species isolated from the studied, where a highly significant difference was obtained ($\chi^2 = 27.5601$; $p<0.05$). These results coincided with different investigations that show *A. flavus* as the most isolated of species in feeds for farmyard birds in a number of different countries (Dalcero *et al.*, 1998; Magnoli *et al.*, 1998, 2005; Pacin *et al.*, 2003; Labuda and Tančinová, 2006; Rosa *et al.*, 2006; Fraga *et al.*, 2007). The importance of this species stems for its capacity to produce and accumulate certain toxic metabolites, such as aflatoxins, kojic acid, aspergillic acid and cyclopiazonic acid (FAO, 2003) The aflatoxins are considered to be the most dangerous toxic metabolites produced by *A. flavus*, both from a toxicological viewpoint and from their frequency of isolation. The aflatoxin B_1 is carcinogenic to humans and is one of the most powerful agents known for causing

Table 1: Frequency of moulds isolated from poultry feed

Genera	Frequency (%)
Aspergillus	36
Penicillium	20
Cladosporium	14
Eurotium	10
Fusarium	4
Syncephalastrum	4
Rhizopus	2
Curvularia	2
Paecilomyces	2
Acremonium	2
Aureobasidium	2
Geotrichum	2

*** = highly significant; $\chi^2 = 17,1668^{***}$

Table 2: Frequency of isolation of fungal species from poultry feed

Species	Frequency (%)
Aspergillus flavus	18
Aspergillus terreus	8
Penicillium citrinum	12
Eurotium herbariorum	10
Cladosporium sp.	6
Penicillium sp.	6
Aspergillus sp.	4
Aspergillus niger	4
Cladosporium macrocarpum	4
Cladosporium herbarum	4
Syncephalastrum racemosum	4
Fusarium moniliforme	4
Aspergillus ochraceus	2
Aspergillus versicolor	2
Aspergillus glaucus	2
Cladosporium sphaerospermum	2
Penicillium purpurogenum	2
Penicillium digitatum	2
Penicillium decumbens	2
Rhizopus oryzae	2
Curvularia lunata	2
Paecilomyces variotii	2
Acremonium butyri	2
Aureobasidium pullulans	2
Geotrichum candidum	2

*** = highly significant; $x^2 = 27,560^{***}$

cancer of the liver (IARC, 1993). Contamination by aflatoxins has been linked to the increase in mortality in domestic and farm animals and with major economic losses for animal feed producers. It has also been seen that in farmyard birds, when they do not cause death, the aflatoxins have hepatoxic and carcinogenic effects and can result in general alterations leading to weight loss, alterations in the food conversion process and immuno-suppression which increases susceptibility of birds to viral and bacterial illnesses (D`Mello and Macdonald, 1997; Benneth and Klich, 2003; FAO, 2003; FAO, 2004; Murphy *et al*., 2006). *Aspergillus terreus* is also commonly found in soils, spices and fodder and is capable of producing toxic metabolites such as citrinine, patulin, citreoviridine and sterigmatocystin (Labuda and Tančinová, 2006).

On the other hand, *A. niger* and *A. ochraceus* were isolated with low frequency in this investigation. However, both were greatly significant for their capacity to produce OTA. In studies carried by Dalcero *et al.* (1998) it was seen that 46% of *A. niger* strains were capable of producing OTA at levels of 13 to 25 ng mL^{-1} in animal feeds in Argentina. Equally, Rosa *et al.* (2006) found that 24% of isolated *A. niger* strains for farmyard birds in Brazil were capable of producing OTA in concentrations of between 10 and 26 μg kg^{-1}. These results suggest that there is a high percentage of ochratoxic strains of *A. niger*, constituting a major source of contamination of animal feeds in South America.

Aspergillus ochraceus is common in food products such as coffee, spices and some cereals and is capable of producing high quantities of toxic metabolites among which are included: OTA, penicillic acid, xantomegnin, viomellein and vioxantin. OTA is considered to be the most significant and most dangerous mycotoxin produced by this species of fungus and it has been classified as a possible human carcinogen. The organisms that are most sensitive to the action of OTA are the kidneys and the liver, although neurotoxic and teratogenic effects and effects on the immune system have also been observed by López *et al.* (2007).

Several studies have made it possible to show the capacity of some strains of *A. ochraceus* for producing OTA in feeds for farmyard birds. Similarly in investigations carried out by Labuda and Tančinová (2006), it was seen that 3 out of 7 of the strains of this species studied were capable of producing OTA in bird feed in the Slovak Republic, while on the American continent, Rosa *et al.* (2006) were able to show that 19 strains of the 74 found in bird feeds in Brazil were capable of producing this mycotoxin in concentration of between 53 and 116 μg kg^{-1}.

Of the *Penicillium* species studied, *P. citrinum* occurred with the greatest frequency (12%). This species is characterized for being omnipresent and for that reason it has been isolated from almost all types of food product. However, the most common sources are milled grain, flour and whole grain cereals, especially rice, wheat and corn. Citrinine is the only mycotoxin produced by *P. citrinum,* which is characterized by a serious renal toxin which, in the case of chickens, may also cause an increase in the consumption of feed, watery diarrhea and weight loss. The permitted limits of this mycotoxin are not known in details given that very few countries have officially regulated them (FAO, 2004).

Eurotium herbariorum has a worldwide distribution, but is found predominantly in tropical and subtropical areas in soils, grains, fruits, fruit juices, dehydrated products, spices and meat products. The species belonging to the *Eurotium* have been described as being extremely xerophyllic and very resistant to high temperatures (Fraga *et al*., 2007). Some researchers have demonstrated the capacity of some of the species of *Eurotium*, among them *E. herbariorum* and its anamorph, *A. glaucus* (found in around 2% of this investigation), to produce OTA, especially in warm and tropical climates (Dalcero *et al*., 2002; Benneth and Klich, 2003). It has also been shown that some species of *Eurotium* are producers of sterigmatocystin and that *E. chevalieri* is capable of producing aflatoxins (Fraga *et al*., 2007; Labuda and Tančinová, 2006).

Table 3 shows the range of concentrations and the percentage of OTA in concentrated feed samples for meat poultry, where 94% of the samples were found to be contaminated with the mycotoxin. The range of contamination was between 2.558 and 31.978 μg kg^{-1}, with the greatest concentration (42%) in the range 10 to 20 μg kg^{-1}. These results were similar to those reported in Brazil by Rosa *et al.* (2006) and it was found that the feed samples for farmyard birds analyzed showed high percentages of OTA contamination at levels which varied between 1.3 and 80 μg kg^{-1}. Results found by Fraga *et al.* (2007), also in Brazil, were similar, indicating that feed samples for farmyard birds show OTA contamination at 100% at concentrations which varied between 17 and 197 μg kg^{-1}. Similarly, the presence of OTA in poultry

Table 3: Range of OTA concentration and percentage of the analyzed samples OTA concentration (μg kg^{-1}), No. of samples (%)

Concentration (μg kg^{-1})	No. of samples	%
0	3	6
< 5	5	10
5-10	14	28
10-20	21	42
20-40	7	14

*** = highly Significant; $\chi^2 = 6{,}000^{***}$

feed ingredients was studied in India by Thirumala-Devi *et al.* (2002) and it was indicated that samples of sorghum and millet showed maximum concentrations of 400 and 145 μg kg^{-1}, respectively; however, the mycotoxin was not detected in samples of corn. These results showed that the presence of OTA was found at levels higher than the upper limits permissible in India (30 μg kg^{-1}).

The FAO (2004) indicates that only 37 countries have regulations for OTA in human and animal food products and indicate further that the limits for this mycotoxin on a worldwide scale in cereals and cereal-based products fall within a range of between 3 and 50 μg kg^{-1}. However, most of these countries (29 in fact-almost all of them belonging to the European Union), have adopted the value of 5 μg kg^{-1} as the maximum permitted limit. In Venezuela, current regulations only consider limits for aflatoxins and these should not be present at levels greater than 0.02 ppm, while the limits for OTA and other mycotoxins is not known and this makes control and monitoring of mycotoxin contamination in human and animal food products more difficult.

On the other hand, in this study a high frequency and contamination by OTA was found in the samples analyzed, which contrasts with the low count of moulds and the low frequency of ochratoxigen species found, such as *E. herbariorum* (10%), *A. niger* (4%), *A. ochraceus* (2%) and *A. glaucus* (2%). These results can be owed to the granulation process, which produces pellets by milling, pressing and heating the fodder, resulting in an insignificant reduction in mycotoxins due to the great stability that they have in conditions of high temperatures in dry substrates (Fraga *et al*., 2007). In fact, we have been able to show that even temperatures of over 250°C are not high enough for the complete destruction of OTA. Furthermore, it has been demonstrated that the half life of this toxin in dry and wet cereals was 707 and 145 min, respectively, at 100°C, showing its strong resistance to high temperatures, mainly in dry substrates (Boudra *et al*., 1995).

On the other hand, the low count of moulds in opposition to the elevated presence of OTA found in this research, suggests that contamination by fungi comes from the raw materials or the initial phases of the production process. This leads to the supposition that perhaps the cereals used in the manufacture of this feed were the main source of fungal contamination and therefore of the high presence of OTA found in this study.

These results allow us to show once again the importance of establishing regulations, especially in developing countries, to guarantee the quality of foods consumed by animals and humans and to protect the economics interests of the industries involved in this field.

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