AFLATOXINS IN MIXED FEEDS FOR RABBITS

Adriana Saubois & Marcelo C. Nepote
Microbiology Laboratory, Biotechnology Department,
Chemical Engineering Faculty
Universidad Nacional del Litoral.
Santiago del Estero 2829, 3000 Santa Fe, Argentina.

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Palabras clave: Aflatoxinas, Aspergillus flavus, alimentos balanceados, conejos.

SUMMARY.
Ten samples of mixed feeds and one of lucern (alfalfa) were analysed. They were supplied by processing plants from Santa Fe Province, Argentina, and designed for feeding rabbits. Several breeding farms had claimed that these lots were the possible cause of intoxication. The degree and type of fungal contamination and the presence of the following mycotoxins were analysed: aflatoxins (AF), ochratoxin A, citrinin, penicillic acid, zearalenone (ZEA), and trichothecenes T-2 toxin, diacetoxyscirpenol (DAS) and deoxynivalenol (DON). The total fungal count was carried out by the dilution plating method, on dichloran rose bengal chloramphenicol agar (DRBC). Isolation of species was performed by means of direct plating on moist chambers as well as the following media: Aspergillus flavus-parasiticus agar (AFPA), chloramphenicol-potato-dextrose agar (PDAC) and dichloran 18% glycerol agar (DG18). Detection and quantification of mycotoxins were performed by means of thin layer chromatography (TLC). It is not possible to assure that the intoxication cases observed by the breeders were caused by mycotoxins. However, the aflatoxins present in 7/11 samples and the high levels found in two of them (200 and 300 μg/kg) could have been the reason.

INTRODUCTION.
Mycotoxins are secondary metabolites of filamentous fungi (especially Hyphomycetes), that can be naturally found in food and feedstuffs. So far, over 200 mycotoxins are known, produced by more than 150 different fungi (1, 2). However, a relatively small number of mycotoxins have been recognized as natural contaminants, namely aflatoxins (AF), ochratoxin A, penicillic acid, and toxins of Fusarium zearalenone (ZEA) and trichothecenes -T-2 toxin, deoxynivalenol (DON), nivalenol (NIV), and diacetoxyscirpenol (DAS)- which have occurred most frequently (14, 21, 36, 38, 39).

Mycotoxicoses diseases caused by mycotoxins-
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include pathological effects and undesirable physiological responses in humans and animals as well, for they affect different organs and tissues. The various toxicity types depend upon dose, organ involved, sex, age and species of the affected animal. They involve acute and chronic effects, hepatotoxicity, nephrotoxicity, hematoxicity, neurotoxicity, dermatotoxicity, gastotoxicity, estrogenism, mutagenicity and cancer (2, 20, 21, 38, 39). Aflatoxins, which are primarily hepatotoxic, are the most strong among those that are known, and can cause severe effects in laboratory animals after an exposure of even a very low level (6, 20, 38). On the other hand, long-term research on farm animals, has proved the existence of other phenomena linked to the exposure to most mycotoxins: a decrease of growth rate, reduction of feeding efficiency and immunosuppression. All these are important economic factors when considering animal breeding (6).

Since their discovery and up to now, there have been few reports regarding field diseases associated to mycotoxins (20). The most characteristic case was the outbreak linked to aflatoxins, which killed 100,000 turkeys ("Turkey X Disease") in England, in 1960 (1). Some other cases were reported afterwards, related to field diseases induced by aflatoxins in ducklings, swine, calves, dogs and trout (38). In European countries there were nephropaties in swine, induced by ochratoxin A (19, 39). Outbreaks linked to T-2 toxin in poultry, horses, livestock and swine, and emetic syndrome in swine associated to DON were reported in USA (39). Estrogenic syndrome in swine and fertility reduction in livestock due to exposure to zearalenone were reported from Europe, USA and Australia (19).

Considering all that has been stated above, rabbit feeds manufactured in Santa Fe, Argentina, were mycotoxicologically analysed. The animals which had been affected, presented hepatic symptoms and a decrease of weight gain. Aflatoxins, ochratoxin A, citrinin, penicillic acid, ZEA, T-2 toxin, DAS and DON were determined in the samples. The mycota present was quantitatively tested and the isolated fungi were identified as regards genus and species. Finally, an association between the potentially toxigenic species and the presence of mycotoxins in the analysed samples was attempted.

**MATERIALS AND METHODS**

Ten samples of mixed rabbit feeds and one of dried ground lucern (alfalfa), used as an ingredient, were analysed. Every feeds corresponded to prestarter feed, starter feed, fattening feed and final feed. The samples were supplied by processing plants from Santa Fe, Argentina, immediately after manufacturing and marketing, from April to October 1990. They were processed for mycotoxicological analysis between the second and the tenth days following their reception at the laboratory. They were kept at 0°C during that period, hermetically, to avoid humidity reabsorption.

a. Sample preparation for mycological analysis:

a.1. Sample preparation for dilution plating method: Portions of 25 g were aseptically taken from each of the 500/800 g total weight samples. The first dilution was prepared in 225 ml of 0.1% aqueous pepto-ne (v/v), shaken in a stomacher for 2 minutes at room temperature. Successive dilutions were obtained from this first one, in the same diluent. Aliquots of 0.2 ml were poured, in duplicate, onto the surface of the culture medium respectively, so as to be counted and isolated (4, 17, 18).

a.2. Sample preparation for direct plating: 25 g portions of the products were sterilized on surface for 2 minutes by means of 10% commercial chlorine bleaching agent and washed several times with sterilized distilled water. Sterilized absorbent paper was used to dry the material before pouring it on sterile moist chambers (two-layer strata of cotton + filter paper).

b. Mycological analysis:

b.1. Total fungal count: Dichloranrose Bengal chloramphenicol agar (DBRC) (16, 28) was used. Plates were incubated at 25°C for 5 days. Counts were expressed as colony forming units per gram (CFU/g).

b.2. Isolation and identification of species:

b.2.1. Dilution plating method: Dilutions were poured on chloramphenicol-potato-dextrose agar (PDAC) (17), on Aspergillus flavus-parasiticus agar (AFPA) (27, 28), and dichloran-18% glycerol agar (DG18) (12), the latter being used for the satisfactory isolation of Eurotium species. Plates were incubated at 25°C up to the appearance of colonies, i.e. 3 to 5 days. Then they were kept at room temperature (22-27°C), daylight incidence being permitted so as to favor the formation of reproductive structures.

b.2.2. Direct plating: Material treated with chlorine bleaching was placed on moist chambers mentioned above. Three dishes per sample were arranged with 5 to 7 particles of material on each one.

The fungi isolated according to the techniques
stated above were placed on PDA for the identification of Fusarium, related specia and dematiaceous hyphomycetes (8, 23, 28, 29), and on Czapek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar to identify other specia (7, 8, 26, 28-30).

c. Mycotoxin analysis:

Goliński et al. multi-method (10) was employed to analyse ochratoxin A, citrinin and penicillic acid. For aflatoxins and ZEA, the BF modified toluene technique was used, according to the Argentinian IRAM standards (24). To analyse trichothecenes techniques employed were that of Kamimura et al. (15) and that of Trucksess et al. for DON in particular (35). Toxin detection and confirmation was carried out by thin layer chromatography; quantification being made by means of visual comparison against the corresponding reference standards.

RESULTS

Table 1, shows the outcome of the mycological analysis. Fungal counts range from 9.0x10² to 3x10⁴ CFU/g. Four samples contained a wider diversity of species, some of them considered "field species" (Acremonium strictum, Epicoccum purpurascens, Cladosporium cladosporioides, Phoma eupyrina, Mucor sp.). Aspergillus flavus was isolated from all samples, including the one of dry ground lucern (alfalfa).

From all the analysed toxins, only AFI B1 and AFI B2 were detected. Table 2, shows the results of this analysis. Four out of eleven samples were free from aflatoxins in detectable levels. Two samples contained both AFI B1 and AFI B2 in levels ranging from traces to 8 µg/kg. Two samples contained AFI B1 in a high level: 200 and 300 µg/kg.

Table 1: Total fungal counts and species detected in different categories of rabbit mixed feeds.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Total Fungal Count (CFU/g)</th>
<th>Fungal Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5x10³</td>
<td>Aspergillus flavus Link: Fr. A. terreus Thom: Penicillium dulciuxii Delacr.: Acremonium strictum W. Gams: Epicoccum purpurascens Ehremb. ex Schech.: Rhizopus stolonifer (Ehremb. ex Link) Lind; Mucor sp.</td>
</tr>
<tr>
<td>2</td>
<td>9.0x10²</td>
<td>A. flavus Link: Fr; Eurotium herbariorum (Wiggers) Link ex Gray</td>
</tr>
<tr>
<td>3</td>
<td>10'&lt;</td>
<td>A. flavus Link: Fr; P. dulciuxii Delacr.; R. stolonifer (Ehremb. ex Link) Lind.</td>
</tr>
<tr>
<td>4</td>
<td>10'&lt;</td>
<td>A. flavus Link: Fr; P. dulciuxii Delacr.; R. stolonifer (Ehremb. ex Link) Lind.</td>
</tr>
<tr>
<td>5</td>
<td>10'&lt;</td>
<td>A. flavus Link: Fr; P. dulciuxii Delacr.; R. stolonifer (Ehremb. ex Link) Lind.</td>
</tr>
<tr>
<td>6</td>
<td>3.0x10⁴</td>
<td>A. flavus Link: Fr; A. terreus Thom; P. citrinum Thom; Cladosporium cladosporioides (Fres) de Vries; E. purpurascens Ehremb. ex Schech.; R. stolonifer (Ehremb.ex Link) Lind.</td>
</tr>
<tr>
<td>7</td>
<td>2.0x10⁴</td>
<td>A. flavus Link: Fr; A. terreus Thom, A. niger van Tieghem, A. candidus Link; P. purpurigenum Stoll, P. expansum Link ex Gray; A. strictum W. Gams.</td>
</tr>
<tr>
<td>8</td>
<td>10'&lt;</td>
<td>A. flavus Link: Fr; A. terreus Thom; P. dulciuxii Delacr.; R. stolonifer (Ehremb. ex Link) Lind.</td>
</tr>
<tr>
<td>9</td>
<td>10'&lt;</td>
<td>A. flavus Link: Fr; A. terreus Thom; P. dulciuxii Delacr.; R. stolonifer (Ehremb. ex Link) Lind.</td>
</tr>
<tr>
<td>10</td>
<td>2.0x10³</td>
<td>A. flavus Link: Fr; A. terreus Thom, A. niger van Tieghem; P. citrinum Thom; Fusarium moniliforme Sheldon; A. strictum W. Gams; C. cladosporioides (Fres) de Vries; Phoma eupyrina Sacc.; R. stolonifer (Ehremb. ex Link) Lind.</td>
</tr>
<tr>
<td>11</td>
<td>10'&lt;</td>
<td>A. flavus Link: Fr; P. citrinum Thom, P. corylophilum Dierckx.; Geotrichum candidum Link ex Leman; C. cladosporioides (Fres) de Vries; E. amstelodami Mangin.</td>
</tr>
</tbody>
</table>

Note:

Feeds: 1-3: Pre-starter feed; 4-7: Starter feed; 8-9: Fattening feed; 10: Final feed; 11: Dry ground lucern (ingredient).

CFU/g: Colony forming units per gram.
Table 2: *Aspergillus flavus* and aflatoxins present in various categories of rabbit feeds.

<table>
<thead>
<tr>
<th>FEED</th>
<th><em>Aspergillus flavus</em></th>
<th>AFLATOXINS (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Link:Fr</td>
<td>AFI B1: tr</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>AFI B2: tr</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>AFI B1: tr</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>AFI B1: 200</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>AFI B1: 5</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>AFI B1: 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AFI B2: 4</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>AFI B1: 300</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>AFI B1: 5</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Note:

Feeds: 1-3: Pre-starter feed; 4-7: Starter feed; 8-9: Fattening feed; 10: Final feed; 11: Dry ground lucern.


DISCUSSION

The huge research work carried out in the USA (1981) and in Poland (1982 and 1985) led scientists to set up warnings regarding fungal counts in mixed animal feeds of 100,000 CFU/g (4). This figure is taken into account when it refers keeping quality in the commercial sense. However, it can be concluded that when figures represent fungal species with health risk for animals, they are also linked to the biological and nutritional quality of the feeds. The samples examined gave maximum counts of 10^4 CFU/g, being the level below the standard of reference, but in all of them species of recognized potential toxigenicity, such as *Aspergillus* and *Penicillium* species were detected and identified (5, 9, 11). The only association observed between toxigenic species and their toxins was the simultaneous presence of *A. flavus* and AFI B1 and AFI B2 in 7 and 2 out of 11 samples, respectively. There is a variety of toxic secondary metabolites that can be synthetized in lab cultures by many of the species from the mentioned genera above (2, 5, 14, 21, 31, 39). However, a precise prediction of their behaviour in food and feeds is not possible. This is due to the fact that these are complex ecosystems influenced by a number of factors namely substratum matrical effect, fungal metabolite interaction (including their synergistic and antagonistic effects) among them and with other organisms, environmental factors (temperature, water activity, CO2/O2, pH) and the external agents such as pesticides (3, 6, 22, 32).

The presence of AFI B1 alone, or AFI B1 and B2 in feeds could have caused the symptoms observed in the animals, even when these toxins had been found in trace levels, since rabbits are one of the most sensitive animal. It has been observed that rabbits and ducklings are the most sensitive to the action of aflatoxins, with an oral LD50 of only 0.3 mg/kg body weight for AFI B1 (20, 38).

Taking into account that mycotoxins -especially aflatoxins- can be heterogeneously distributed in feed matrixes (13), it is possible to state the hypothesis that suspicious feeds (those in which aflatoxins were not detected) could have been not conveniently sampled and that the samples taken for the analysis were not representative of the portion eaten by the animals. When food or feed is kept in a wrong way, mainly under humidity and temperature conditions that favour fungal development and the production of mycotoxins, the latter can develop in certain points and not in the total volume of the lot. Some years ago it was proved that these toxins can be found in the fields, when *A. flavus* invades peanut, cottonseeds and corn under certain conditions (6, 18, 22, 34). The bibliography indicates that, even in those products which undergo intensive mixing, the distribution of aflatoxins is heterogeneous enough to allow variations in the contamination levels reach two orders between two different portions of the same lot (25). The presence of *A. flavus* in all of the samples places them in the category of "suspicious feeds". Nowadays, over 40 countries -including the EEC- have current or proposed legislation for the regulation of aflatoxins in animal feeds. Several countries (mainly those which need to import raw material or manufactured products) have quite high tolerance levels. These values are almost exclusively applied to certain ingredients of mixed feeds that, in
turn, must be mixed so as to obtain the final product. Thus, an important reduction of the initial contamination is achieved (from peanut, soya and cotton seeds by-products, mainly). The type and age of the animals to be fed are also taken into account. In China, the maximum tolerance is of 1,000 µg/kg for total aflatoxins in feedstuffs ingredients, with a maximum of 4% in final products. In Japan, the maximum tolerance is of 1,000 µg/kg of Afl B1 for import peanut meal (maximum: 2 to 4% of this material in final products). The EEC accepts a maximum of 200 µg/kg of Afl B1 in certain ingredients. France and Senegal allow 300 µg/kg of Afl B1 in general ingredients and peanut based feedstuffs respectively. Nevertheless, most countries have set a maximum tolerance level ranging from 10 to 50 µg/kg for both, Afl B1 and the total aflatoxins in final products, exceptions being not higher than 100 µg/kg (33, 37). From all that has been stated above, it is considered that Afl B1 values of 200 µg/kg in prestarter feed and 300 µg/kg in fattening feed detected in this work are alarming levels, taking into account the fact that they are final products for animal consumption, especially when these animals are highly susceptible as rabbits are.

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REFERENCES


Aflatoxins in mixed feeds for rabbits - A. Saibois & M.C. Nepote


