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Mycoflora and Aflatoxins Associated with Poultry Feeds in Aseer Region, Saudi Arabia

الفطريات والسموم الفطرية (الأفلاتوكسينات) المصاحبة لعلف الدجاج في منطقة عسير بالمملكة العربية السعودية

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Abstract: One hundred and twenty feed samples collected from different poultry farms located in Aseer region of Saudi Arabia were screened from January to December 2003. Twelve fungal genera and 27 species were identified. *Aspergillus* (85%) and *Penicillium* (47%) were the genera most commonly isolated from the feeds. Aflatoxins B1, B2, G1 and G2 were produced by twelve fungal species. *Aspergillus flavus* and *A. parasiticus* produced the largest amount of aflatoxin B1 (700 and 500 Ìg/l, respectively). *A. parasiticus* produced the largest amount of aflatoxins B2 and G2 (400 and 300 Ìg/l, respectively). *Penicillium notatum* produced the highest amount of aflatoxin B2 (140 Ìg/l). *Scopulariopsis brevicaulis* produced the largest amount of aflatoxin G1 (280 Ìg/l). Propionic and oxalic acids completely inhibited the growth and aflatoxin production of *A. flavus* and *A. parasiticus* between pH 2.5 and 5.0. Citric and tartaric acids, on the other hand, completely inhibited the growth and aflatoxin production of the same species at pH values between 2.5 and 4.0.

Key words: Aflatoxins, mycoflora, poultry feeds, Saudi Arabia.

المستخلص: تم جمع 120 عينة من علف الدجاج من مزارع مختلفة في منطقة عسير بالمملكة العربية السعودية في الفترة من يناير إلى ديسمبر 2003 م وذلك لمعرفة الفطريات والسموم الفطرية (الافلاتوكسينات) المصاحبة لهذا العلف. تم عزل وتعريف 27 نوعا من الفطريات تنتمي إلى 12 جنسا من عينات العلف. وكانت أجناس *Aspergillus* و *Penicillium* هي الأكثر شيوعا في العلف حيث سجل الأول نسبة 85 % والثاني 47 % من مجموع الأجناس الفطرية. كما أوضحت النتائج أيضا أن الأفلاتوكسينات B1, B2, G1, G2 أنتجتها 12 نوعا من الفطريات. وقد وجد أن نوعي *Aspergillus flavus* و *Aspergillus parasiticus* برازيتيكس أنتجا أكبر كمية من الأفلاتوكسين B1 (700 و500 ميكروجرام/لتر وسط غذائي بالترتيب). كما وجد أن *A. parasiticus* أنتج أكبر كمية من الأفلاتوكسينات B2 و G2 (400 و 300 ميكروجرام/لتر وسط غذائي بالترتيب) . أما *Penicillium notatum* فقد أنتج أكبر كمية من الأفلاتوكسين B2 (140 ميكروجرام/لتر وسط غذائي) . وكان *Scopulariopsis brevicaulis* قد انتج أكبر كمية من الأفلاتوكسين G2 (280 ميكروجرام/لتر وسط غذائي). كما أوضحت النتائج أن حمضي الأوكساليك والبروبيونيك قد منعا تماما نمو وإنتاج الأفلاتوكسينات لكل من *A. flavus* و *A. parasiticus* وذلك عند مدى pH يتراوح بين 2.5 4 وذلك بخلاف حمضي التارتاريك والسيترك اللذين منعا نمو نفس الفطرين بنسب متفاوتة عند مدى pH يتراوح بين 2.5 - 5.

كلمات مدخلية: الأفلاتوكسين، الفطريات، علف الدجاج، المملكة العربية السعودية.

Introduction

The presence of fungi and aflatoxins in poultry feeds result from the raw material used in their production. Fungi and aflatoxins contamination of the raw materials occur during the pre-harvest (field-produced fungi) and /or the post-harvest (storage-produced fungi) periods. During these periods, temperature and humidity play an important role in the growth of fungi and aflatoxins contamination (Lozada, 1995). Mycotoxins, particularly aflatoxins pose a significant threat to both human and animal health because they are toxigenic, carcinogenic, mutagenic and teratogenic (Hsiehd, 1986; Chu, 1997), besides the economic loss due to food contamination. Aflatoxins are structurally related compounds produced as secondary metabolites by toxigenic strains of *Aspergillus flavus, A. parasiticus and A. nomius* (Betina, 1989). As a recognized health hazard,

aflatoxins are the most important among the known mycotoxins (Scott, 1973). All poultry species are susceptible and more sensitive to aflatoxins (Dalcero, *et al.* 1998).

In Saudi Arabia, there is very little information about this topic, except within the work of Ewaidah (1989), who reported the survey of aflatoxins in poultry feeds in Riyadh region. The purpose of this study was to investigate the mycoflora composition in stored poultry feeds and the ability of these fungi to produce aflatoxins. Additionally, the effect of some aliphatic acids on fungal growth and aflatoxins production was investigated.

Materials and Methods

1. Isolation of fungi

The poultry feed used in this study was obtained from different poultry farms in Aseer region of Saudi Arabia. One hundred and twenty samples were screened from January-December 2003. The feed was assayed monthly for fungal content using the method described by Dalcero, *et al.* (1998). Each month, six plates of Czpapek's agar medium with streptomycin (20 µg/ml) and rosebengal (30 ppm) as bacteriostatic agents (AL-Doory, 1980) were inoculated and incubated at 28°C for 7-9 days. The developing fungi were identified and counted.

2. Identification

Purified fungal isolates were identified, whenever possible, in the original Petri dish culture according to published procedures (Raper and Fennell, 1965; Ellis, 1971; 1976; Samson, 1979; Domsch, *et al.* 1980; Ramirez, 1982; Nelson, *et al.* 1983; Pitt, 1985).

3. Determination of moisture content

Two g of five replicates were dried with forced air circulation for 16 hours at 80°C to constant weight. The moisture content was calculated as a percentage on an oven dry basis.

4. Evaluation of aflatoxins in poultry feeds

This procedure was based on Richard, *et al.* (1993). Fifty g of feed were mixed and homogenized in a mechanical blender with 50 ml of 55% methanol and 100 ml petroleum ether, then blended at high speed for one minute followed by centrifugation at 2000 rpm for five minutes. Twenty-five ml of the methanol phase were extracted, purified and subjected to chromatographic analysis as mentioned before.

5. Production of aflatoxins by fungi in liquid medium

Production of aflatoxins in yeast extract-sucrose (YES) liquid medium was carried out according to Harwig *et al.* (1979). Fungal spores were inoculated in 50 ml medium, incubated under static conditions at 28°C for seven days, followed by determination of the dry weight of the mycelia.

6. Extraction of aflatoxins

Fifty ml of filtrates were mixed with an equal volume of chloroform, and the extracts were concentrated as described by Epply (1968). The extracts were then purified by silica gel column chromatography (Jones, 1972). The purified aflatoxin extracts were concentrated in a rotary evaporator; the residues were dissolved in 1 ml of chloroform. The extracts were used for screening by TLC or the bioassay test.

7. Thin layer chromatography (TLC)

TLC was carried out according to Richard *et al.* (1993) by spotting 25 µl of chloroform extracts against aflatoxins B1, B2, G1 and G2 standards on 20 x 20 cm plates covered with 0.5 mm silica gel. The plates were developed in toluene: ethyl acetate: formic acid (60: 30: 10 v/v/v). The plates were examined under long wave UV light (366 nm). The quantity of aflatoxins was determined by flourodensitometry at 336 nm as described by Schuller and Van Egmond (1983).

8. Bioassay of aflatoxins

For aflatoxin bioassay, brine shrimp (*Artemia salina* L.) larvae were used according to the method described by Korpinen (1974). Twenty µg of the previous extracts were added to 2 ml seawater containing 60-100 larvae. After 24 hrs, the percentage of mortality was determined.

9. Inhibition of aflatoxin production

Inhibition of aflatoxin production by tartaric, citric, oxalic and propionic acids was determined according to the method of Tong and Draughon (1985).

Table 1. Distribution of mycoflora in 120 samples of poultry feeds

Genera and Species	Frequency %	Genera and Species	Frequency %
Aspergillus	85	*Mucor circinelloides*	4
Aspergillus flavus	45	*Absidia glauca*	5
A. parasiticus	33	*Rhizopus nigricans*	5
A. fumigatus	2	*Cunninghamella echinulata*	4
A. niger	1	*Aphanomyces laevis*	3
A. ochraceus	1	*Cephalosporium*	7
A. sydowi	1	*Cephalosporium curtipes*	4
A. terreus	1	*C. roseo-griseum*	3
A. wentii	1	*Cladosporium*	5
Penicillium	47	*Cladosporium cladosporides*	2
Penicillium notatum	30	*C. sphaerosporium*	3
P. oxalicum	9	*Alternaria*	5
P. implicatum	3	*Alternaria alternata*	3
P. roqueforti	4	*A. cheiranthi*	1
P. brevicompactum	1	*A. brassiciola*	1
Scopulariopsis brevicaulis	5	*Syncephalastrum racemosum*	2

Results and Discussion

● Mycoflora of stored poultry feed

The count of isolated fungi was defined as the percentage of samples in which each fungus was present. During the sampling period, 27 species belonging to 12 genera were recovered from the stored poultry feed (Table 1). The species belonging to *Aspergillus* (85%) and *Penicillium* (47%) genera were the most frequent. These genera were represented by a variety of species. *Aspergillus* was the most common genus and eight species were identified. Among *Aspergillus* spp., *A. flavus* and *A. parasiticus* were the most frequent (45 % and 33 %, respectively) (Table 1). *Penicillium* was the second most frequent genus and five species were identified. Among *Penicillium* spp., *P. notatum* was the most frequent (30 %) (Table 1.).

Many researchers have proved the majority of feeds has species from *Aspergillus* and *Penicillium* genera as a predominant flora (Dalcero, *et al.* 1998).

● Screening of aflatoxins from stored poultry feed

Fungal counts in the Czpapek's agar medium ranged from 1.2 x 10^2 to 9.5 x10^5 CFU/g. Aflatoxin analysis from these samples, showed that aflatoxin B1 was the one of highest incidence, because it appeared in 62 (52 %) of the samples. Aflatoxin B2 appeared in 27 (23 %), aflatoxin G2 in 18 (15%) and aflatoxin G1 in 14 (12%) of the samples. The levels varied between 10-20, 10-40, 10-70 and 20-100 ng/g for B1, B2, G1 and G2, respectively (Table 2).

Moisture content of the samples ranged from 1.5 and 20 % during the sampling period. High moisture content of feeds was correlated with high counts of fungi (Table 3). Rising fungal counts coincided with low temperature and high humidity, since January is the coldest and most humid month in Aseer region. Apparently, the temperature of this month is convenient for the maximum growth of fungi. In this study the highest level of aflatoxin B1 was detected during January when the registered moisture percentage in the sample was of 20%.

● Production of aflatoxins by fungi in liquid medium

Aflatoxins were produced by 36 isolates of *A. flavus*, which possessed the largest number of aflatoxin-producing strains, which were distributed as follows; 23 isolates produced aflatoxin B1, 9 produced B2, and 4 produced more than one aflatoxin (Table 4). Isolate no. 63 produced the highest amount of aflatoxin B1 (700 Ìg/l) (Table 4). In addition, aflatoxins were produced by 33 isolates of *A. parasiticus,* which distributed as follows; 19 isolates produced aflatoxin B1, 11 produced aflatoxin B2, and 3 produced more than one aflatoxin (Table 5). Isolate no. 439 produced the highest amount of aflatoxin B1 (500 Ìg/l) (Table 5). Other 12 fungal species produced different types of aflatoxins (Table 6). Thirty isolates belonging to other 12 species produced aflatoxins (Table 6). *P. notatum* comprised the majority of aflatoxin-

Table 2. Aflatoxins analysis from 120 samples of poultry feeds

	Aflatoxins levels							
	B1		B2		G1		G2	
Months	% Positive samples [1]	Levels (ng/g) [2]	% Positive samples [1]	Levels (ng/g) [2]	% Positive samples [1]	Levels (ng/g) [2]	% Positive samples [1]	Levels (ng/g) [2]
January	100	120	80	20	50	10	60	80
February	100	14	60	10	60	14	40	100
March	90	10	30	10	10	70	0	ND
April	80	18	20	15	0	ND	0	ND
May	30	80	0	ND	0	ND	0	ND
June	0	ND	0	ND	0	ND	0	ND
July	0	ND	0	ND	0	ND	0	ND
August	20	18	0	ND	0	ND	0	ND
September	0	ND	10	30	0	ND	20	20
October	50	16	0	ND	0	ND	0	ND
November	70	ND	120	18	20	16	40	50
December	80	90	50	40	0	ND	20	40

[1] percentage of positive samples from 10 samples per month.
[2] Means of c oncentration aflatoxins based on positive samples only. ND = Not detected.
Detection limits (ng/g) aflatoxins B1, B2, G1, G2 = 1.

P. notatum produced aflatoxin B1, three produced aflatoxin B2 and five produced G2. Isolate no. 64 of *P. notatum* produced the highest amount of aflatoxin B2 (140 Ìg/l) (Table 6). Moreover, isolate no. 110 of *Scopulariopsis brevicaulis* produced the highest amount of aflatoxin G1 (280 Ìg/l) (Table 6).

These results indicate that Aspergilli were the predominant fungal contaminants and aflatoxin producers. These results were in agreement with the results obtained by pervious workers (Dalcero *et al.* 1998 and Gayakward *et al.* 2001). Also, *A. flavus* was encountered in high frequency in the feed. This fungus is a well-known glucophilic cellulose-decomposer and aflatoxin-producing fungus (Abdel-Hafez and EL-Maghraby, 1992).

Table3. Monthly total count of fungi and moisture content of poultry feeds.

Month	Total count of fungi [1]	Moisture content (%) [2]
January	$9.5 \times 10^5 \pm 0.04$	20.0 ± 0.14
February	$7.2 \times 10^5 \pm 0.05$	15.5 ± 0.25
March	$1.3 \times 10^4 \pm 0.71$	5.0 ± 0.02
April	$6.2 \times 10^3 \pm 0.08$	4.1 ± 0.03
May	$1.6 \times 10^2 \pm 0.11$	3.4 ± 0.05
June	$1.2 \times 10^2 \pm 0.21$	3.0 ± 0.06
July	$1.8 \times 10^3 \pm 0.05$	1.5 ± 0.11
August	$1.7 \times 10^2 \pm 0.12$	6.5 ± 0.14
September	$1.4 \times 10^2 \pm 0.07$	6.4 ± 0.24
October	$1.9 \times 10^2 \pm 0.21$	8.0 ± 0.15
November	$8.3 \times 10^3 \pm 0.13$	7.7 ± 0.07
December	$7.8 \times 10^5 \pm 0.08$	9.6 ± 0.21

[1] Mean mold count (CFU/g). Standard error
[2] Mean percentage moisture. Standard error

- Effect of selected aliphatic acids on the production of aflatoxins

Because *A. flavus* and *A. parasiticus* produced the largest quantity of aflatoxin, these species were selected to study the effect of some aliphatic acids on fungal growth and aflatoxin production. Propionic and oxalic acids completely inhibited growth and aflatoxin production of both species at the pH values tested. On the other hand, tartaric acid inhibited aflatoxin production by both species. *A. flavus* produced a small amount of aflatoxin B1 at pH 4.5 when compared with the control (Table 7). Citric acid inhibited growth and aflatoxin production by both species at pH 2.5 and 4.0. However, at pH 4.5 and 5.0 the production of aflatoxin B1 by *A. flavus* was dramatically reduced and the production of aflatoxins G1 and B2 by *A. parasiticus* was not affected by citric acid.

Beckwith *et al.* (1975) reported that aqueous solutions of strong acids have the ability to destroy the biological activity of aflatoxins B1 and G1. Vandegraft *et al.* (1975) found that 1% propionic

Table 4. Aflatoxins produced by *A. flavus* isolated from stored poultry feeds on yeast extract-sucrose medium

Isolation month	Strain number, type and quantity (µg/l) of aflatoxin
January	1(B1,40), 3(B1,120), 5(B2,G2,140,20), 29(B1,80), 45(B1,40), 182(B2,40), 190(B2,20)
February	51(B1,700), 54(B1,160), 72(B1,160), 78(B1,120), 80(B2,140)
April	88(B1,120), 89(B2,B1,180,20), 90(B2,20)
May	125(B2,G2,60,20), 126(B1,160), 131(B1,40), 132(B2,140)
June	191(140), 218(B1,40), 220(B1,20)
July	246(B1,80), 249(B2,20), 268(B1,140)
August	339(B1,G2,160,40), 340(B1,60), 341(B2,120), 355(B1,80)
October	384(B1,140), 391(B1(120)
November	408(B1,40), 410(B2,60)
December	430(B1,120), 432(B2,100), 447(B1,80)

The quantity of aflatoxins are produced from 2 g dry mycelium/100 ml medium

Table5. Aflatoxins produced by *A. parasiticus* isolated from stored poultry feeds on yeast extract -sucrose medium

Isolation month	Strain number, type and quantity (µg/l) of aflatoxin
January	13(B1,40),14(B1,120), 17(B2,G2,140,20), 18(B1,80), 32(B2,400)
February	33(B1,180), 34(B1,160), 37(B2,20), 38(B2,20), 41(B2,160), 44(B1,40)
March	55(B1, 160), 62(B1,120), 211(B1,140), 214(B2,120), 256(B1, B2,180,20)
May	281(B2,G2,60,20), 310(B1,160), 315(B1,40)
June	346(B2,40), 349(B2,20)
July	350(B1,120), 365(B1,100)
August	375(B1,40), 379(B2,60)
October	403(B1,140)
November	425(B1,80), 428(B2,G2,60,300)
December	439(B1,500), 441(B1,140), 444(B2,60), 445(B1,80), 446(B2,140)

The quantity of aflatoxins are produced from 2 g dry mycelium/100 ml medium

acid significantly reduced mold growth and aflatoxin production. The present results indicate that pH plays an important role in the inhibition of aflatoxin. For example, citric acid was completely inhibited fungal growth and aflatoxin production at pH 2.5-4.0 but not at 4.5-5.0. Shin and Martin (1975) reported that the degradation of aflatoxins B1, B2 and G2 by *A. parasiticus* occurred between pH 2.5 and 6.0. The practical use of sodium propionate as a fungal inhibitor was significantly dependent on the pH (Tong and Draughon, 1985). With 0.2% sodium propionate, growth of *A. sulphureus* was inhibited only by 13% at pH 5.5 compared with 100% at pH 4.5.

A mold inhibitor is an excellent part of a management system for the ruminant animal producer (Tabib, *et al.* 1984). When one selects a mold inhibitor it should be one based on more than

Table6. Aflatoxins produced by fungi other than *A. flavus* and *A. parasiticus* isolated from stored poultry feeds on yeast extract-sucrose medium

Isolation month	Fungal species	Strain number, type and quantity (µg/l) of aflatoxin
January	*Penicillium notatum* *Absidia glauca*	22(G2,40),55(B1,120), 64(B2,140), 65(G2,20), 74(G2,80), 82(G2,40), 83(B2,80), 87(B2,60) 95(G2,160)
February	*Scopulariopsis brevicaulis* *Cunninghamella echinulata* *Absidia glauca*	110(G1,280), 116(B2,160) 129(G2,160) 150(G2,120)
March	*Scopulariopsis brevicaulis* *Cunninghamella echinulata*	182(G2,60), 219(G2,40) 230(G2,80)
April	*Absidia glauca*	235(G2,80)
May	*Rhizopus nigricans* *Cladosporium cladosporides* *Cephalosporium roseo-grseum*	240(B2,60) 270(G2,20) 334(G2(160)
June	*Mucor circinelloides* *Rhizopus nigricans*	395(G2,20) 397(B2,160)
July	*Alternaria cheiranthi*	380(G2,140)
October	*Mucor circinelloides* *Cephalosporium roseo-grseum*	395(G2,20) 397(G2,40)
November	*Mucor circinelloides* *Cladosporium cladosporides* *Cephalosporium roseo-grseum*	399(G2,20) 414(B1,40) 429(G2,100)
December	*Syncephalastrum racemosum* *Cladosporium sphaerosporium*	432(B1,40) 440(B1,140)

The quantity of aflatoxins are produced from 2 g dry mycelium/100 ml medium

Table7. Inhibition of growth and aflatoxin production of *A. flavus* (Af) and *A. parasiticus* (Ap) by citric and tartaric acids at pH 2.5 -5.0

Acid	pH	Growth		Aflatoxins (µg/l)								D.W.M.	
				B1		B2		G1		G2			
		Af	Ap	Af	Ap	Af	Ap	Af	Ap	Af	Ap	Af	Ap
Tartaric	5.0	++	-	90	-	-	-	-	-	-	-	1.35	-
	4.5	+	-	20	-	-	-	-	-	-	-	0.69	-
	4.0	-	-	-	-	-	-	-	-	-	-	-	-
	3.2	-	-	-	-	-	-	-	-	-	-	-	-
	2.5	-	-	-	-	-	-	-	-	-	-	-	-
Citric	5.0	++	+	50	-	-	15	-	20	-	-	1.70	0.42
	4.5	+	+	20	-	-	15	-	20	-	-	0.71	0.41
	4.0	-	-	-	-	-	-	-	-	-	-	-	-
	3.2	-	-	-	-	-	-	-	-	-	-	-	-
	2.5	-	-	-	-	-	-	-	-	-	-	-	-
Control	7.5	+++	+++	800	-	-	20	-	240	-	-	2.23	2.57

Oxalic and propionic acids were also tested, and completely inhibited growth and aflatoxins production. +++ = high growth, ++ = moderate growth, + = weak growth, - = no growth or no aflatoxins production.

D.W.M. = Dry weight of mycelium expressed as mg/100 ml medium

one active ingredient. Propionic acid or products containing this acid are widely used to inhibit mold growth (Paster, *et al.* 1987). These products are fungistats and not fungicides; that is, they only inhibit growth of molds and do not inactivate any toxins already present. Mold inhibitors will not keep mold growth in check indefinitely. In general, the salts of the acids last longer, but are not as effective as the free acids; the volatile free acids have greater efficacy but are not long lasting (Tabib, *et al.* 1984). It appeared that the effectiveness of propionic acid as a mold inhibitor can be greatly increased by the pelleting process and that a decrease in the fungal burden of feed.

- Bioassay of aflatoxins.

The bioassay of aflatoxins indicated that isolate no. 51 of *A. flavus*, the isolate no. 438 of *A. parasiticus,* isolate no. 64 of *P. notatum* and the isolate no. 110 of *S. brevicalus* produced the highest amount of aflatoxins and caused the highest mortality of brine shrimp larvae. Moss and Smith (1985) stated that aflatoxin B1 causes chromosomal aberrations and DNA breakage in plant and animal cells. El-Zawahri *et al.* (1977) demonstrated that aflatoxin B1 is a strong chromosome-damaging agent and caused a high rate of breaks and translocations in treated cells.

In conclusion, aflatoxins can contaminate food supply directly or indirectly (Jarvis, 1975). Direct contamination occurs as the result of mold growth on the food material itself. Indirect contamination occurs as the result of using a food ingredient contaminated with aflatoxin residues. Poultry feeds contaminated with aflatoxins may contribute to the accumulation of toxins in poultry and may cause serious health problems to consumers. Aflatoxin B1 is the most potent naturally occurring hepatocarcinogenic agent so far recognized (Bosch and Peers, 1991). Hence, precautions must be adopted during handling, storage and processing of poultry feeds to avoid contamination and serious deterioration by filamentous fungi. Several of these fungi could produce aflatoxins, which are harmful to poultry and subsequently to human health.

The need for controlling mold in ruminant feeding situations is now becoming more evident. Progressive producers will accept these strategies in the years to come.

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