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RESEARCH ARTICLE

Determination of *AFLD* and *AFLQ* Genes Responsible for Aflatoxin Formation in Some livestock Concentrated Feeds

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Investigation of *AFLD* and *AFLQ* genes responsible for aflatoxin formation in concentrated feeds produced in Northern Syria and determination of chemical composition of these feed stuffs were main targets of this study. For this aim concentrated feed samples, from different ruminant and poultry feed suppliers, were collected. Crude protein, crude fat, crude cellulose, and crude ash were determined. Results indicated that the chemical compositions of the feed samples were varied. Aflatoxin levels of the feed samples were also analyzed and all samples were found to be contaminated with aflatoxin at various levels. Two genes responsible for the biosynthesis of aflatoxin B1 were amplified by means of Polymerase Chain Reaction (PCR). The study found that the expression profiles of these two genes were consistently correlated with strain's ability to produce aflatoxins or not in yeast extract-sucrose (YES) agar. The aflatoxin concentrations determined by fluorescence *HPLC-F* were observed to be ranging from 0.1 to 60 Ag/ml of the *AflD*, and *AflQ* culture filtrates. The results are discussed in relation to the suitability of PCR based methodologies where individual isolates are being tested for potential toxin production to identify toxinogenic isolates of *Aspergillus* species.

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INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by various genera of fungi that contaminate crops and processed food and feed stuff all over the World. The most common mycotoxin is Aflatoxin (AF), belongs to family of deketides, is identified as secondary product of mainly the genus *Aspergillus* (*A. flavus, A. parasiticus* and *A. nomius*) often contaminating cereals along the line of their growth, harvest, storage, transport and processing for the production of food or animal feed (Bryden, 2007; Levin 2012; Naseem *et al*., 2018). Aflatoxin B1 (*AFB1*) represents a highly toxic, mutagenic, teratogenicity and carcinogenic compound that exhibits an immuno-suppressive activity, acute and chronic toxicity in both humans and animals exposed to this toxic material (Meggs, 2009; Saleemi *et al*., 2012). Concentrated feeds, highly represented in animal diets particularly for farm animals, might be contaminated by mycotoxin-producing moulds.

The main source of *AFB1* exposure, thereby being concentrated feed, cereals and cereal-based products both for human and farm animals. In cattle fed with contaminated feed by *AFB1* gets to be converted into *AFM1* and subsequently excreted in their milk, and therefore concerns about the entry of mycotoxins into the food chain through meat, milk and particularly into dairy products have been raised in recent decades (Markov *et al*., 2013). The maximum content of *AFB1* in feed materials has been set to 20 mg/kg in the EU regulations (Commission Regulation 574/2011) and lesser amounts are allowed for both *AFB1* and *AFG1* in food and cereals. Production of AF in these commodities takes place during the secondary metabolic activity of the aflatoxinogenic fungi, involving the expression of *aflD* (synonym *Nor-1*) transcript gene (Yu, 2012) which is a determinant gene in the anabolic process of *AFs*.

Molecular methods, alongsight the conventional microbiological techniques, have been applied in recent years for the identification of fungal aflatoxinogenesis with different levels of success (Sweeney *et al*., 2000; Degola *et al*., 2007; Rodrigues *et al*., 2009). PCR-based methods, including monomeric, quadruples and multiplex

PCR have been used vastly to investigate the genes responsible for AF biosynthesis (Somashekar *et al*., 2004; Manonmani *et al*., 2005). Correlating the expression profile of AF biosynthesis genes with aflatoxin production by *A. flavus* and *A. parasiticus* strains using *RT-PCR* and real-time *PCR* have been previously achieved. There are significant correlation between aflatoxin production by *A. parasiticus* and expression of two genes, *aflR* and *aflQ*, as determined by *RT-PCR* in AF conductive and non-conductive media (Sweeney *et al*., 2000). Expression of *aflD*, *aflQ*, and *aflP* genes also provided useful differentiation of aflatoxinogenic and non-aflatoxinogenic strains of *A. flavus* and *A. parasiticus* (Rodrigues *et al*., 2009).

In Syria the compound feed is produced mainly by commercial feed mills and in some extent as homemade mixtures. Manufacturing, distribution and storage of feedstuff are not regulated by law properly, and their production is not under strict control especially after starting of the interior war. All that truths add new problems to the feed quality by causing mycotoxicosis. The owners of feed mills or manufacturers purchase huge amount of these crude feed materials during the production season and store them for making animal feed throughout the year and it is well known that mold growth is associated with improper storage conditions. Major aims of the present study were to isolate, purify and identify the fungi from different concentrated feeds samples collected from various farms and feed factories in Northern part of Syria and moreover to investigate the aflatoxinogenesis levels of these fungal isolates with the aid of molecular methodologies.

MATERIALS AND METHODS

Samples: There are five agro-climatic zones in Syria based on the amount of yearly rainfall. Idlib province located in the Northern part of Syria in the border of Turkey with annual rainfall ranged between 250-600 mm. The rainy season in Syria is mainly between December and March and therefore all samples were collected in December 2014 (n=8), January 2015 (n=6) and February 2015 (n=6). For all analysis 1kg of feed samples (for each collection) collected randomly from factories, farms and stores located geographicly in various parts of Idlib province. A total of 20 samples were collected from the concentrated feeds of cattle (n=5), sheep and goat (n=5) and poultry (n=10). All samples were kept in pre-sterilised naylon bags and brought to Biotechnology and Gene Engineering Laboratory of Animal Science Department, Kahramanmaras Sutcu Imam University and stored at 4^0C until further analysis. Nutirient composition of all samples were analyzed according to AOAC (2001).

Isolation and identification of mould: All samples taken from various kind of concentrated feed were grounded gently and plated on Potato Dextrose Agar (PDA) in duplicates and were incubated at 31°C for 72 h. Fungal colonies on feedstuff which plated on PDA agar were counted and identified at genus level first according to their basic morphological characters as follow; *Fusarium spp.* according to (Pitt *et al*., 2009). In this case the colonies in PDA Broth incubated at 37°C for 2 days, and then the positive samples were transferred and grown in PDA agar to obtain a stock culture. The medium was modified as described by Hepperly *et al*. (1981). Mixture of chloramphenicol and dichloran were used in all culture media to inhibit possible bacterial contamination and growth.

DNA Extraction: Conventional naming system of aflatoxin gene outlined by Yu, (2012) was taken as reference and all samples were tested for the presence of *aflD* and *aflQ* genes. The fungal colonies grown in PDA broth for 3 days were transferred into 2 ml eppendorf tubes containing 2 mm diameter glass beads. Then 650 µl of lysis CTAB buffer was added and the mixture was ground in a tissue miller at a frequency for 30 minutes after which samples were incubated at 65°C for 2 hours. Proteins were precipitated by adding 600 µl phenol. The tubes were inverted gently and centrifuged at 13585 g for 20 minutes. The top aqueous layer was pipetted into new tubes, 600 µl phenol: chloroform (25:24 v/v) was added and inverted gently to get a proper mixture and centrifuged at 13585 g for 20 minutes. Cleaned supernatant was transferred into new 2 ml eppendorf tubes and washed in 60 µl sodium acetate buffer (pH: 8) then 800 µl isopropanol were added gently and incubated overnight at 4°C for precipitation. The samples were centrifuged at 13585 g for 10 minutes in a refrigerated centrifuge (Selecta, Spain) at 4°C, the supernatant discarded after which the DNA pellet was washed twice using 1ml 70% ethanol and centrifuged again at 13585 g for 5 minutes at 4°C. The supernatant was discarded and the DNA pellet dried in an oven at 55°C for 30 minutes. The pellet was re-suspended in 80 µl low salt TE buffer, 5 µl RNase (1mg/ml) was added to remove any possible RNA contamination and stored at -20°C until further usage (Levin, 2012).

PCR procedure: PCR amplification was performed by adding 1 µl dNTP mix, 4 µl Buffer and 0,5 µl Taq DNA polymerase and diluted in 32 µl distilled water. The *aflD* gene was amplified using the primers of *Nor1-F* (5′-ACC GCT ACG CCG GCA CTC TCG GCA C-3′) and *Nor1-R* (5′-GTT GGC CGC CAG CGA CAC TCC G-3′) (Rodrigues *et al*., 2009). The *aflQ* gene was amplified using the primers *Ord-1F* (5′-TTA AGG CAG CGG AAT ACA AG-3′) and *Ord-1R* (5′-GAC GCC CAA AGC GGA ACA CAA A-3′) (Sweeney *et al*., 2000).

Aflatoxin analysis: The fungal isolates which were found to be positive for *Aspergillus flavus* cultured on YES agar (Rodrigues *et al*, 2009) were analysed further for aflatoxin production profile. The isolates were incubated on YES agar containing plates at 28°C for three days and examined under UV light to check the positive and negative isolates for aflatoxin production according to Levin (2012) with slight modification as follow. The samples were filtered through Whatman No.4 and 20 ml of clear filtrate was collected. Then 5 ml of the filtrate was diluted using 120 ml of 0.01 M phosphate buffered saline, it was ensured that 125 ml of diluent equivalent to 1g sample was taken into the glass syringe barrel for passage through the immune affinity column. All extracts were passed through an immune affinity column at a flow rate of 2-3 ml min^{-1}. Sample containing vials were then placed beneath the column and 1 ml of HPLC grade methanol was transferred to the glass barrel. Aflatoxins

were eluted from the column at a flow rate of 1 drop per second. After completion of the election, 1 ml of distilled water was pipetted into the glass syringe barrel and passed through the column to get a final volume of 2 ml (Levin, 2012).

Aflatoxin quantification: Aflatoxin contents were measured using *High-Performance Liquid Chromatography (HPLC-F)*. All the samples were derivatived using trifluoroacetic acid (TFA) according to the standard method reported by Anastassiades *et al*. (2003). An *HPLC* system (Waters 600E) with a fluorescence detector (Waters 470) and an auto-sampler (Waters 712 WISP) were used for aflatoxin quantification and detection. Reference standards (Sigma-Aldrich, USA) were prepared in 50 µl of each of Aflatoxin B1, G1, B2 and G2 containing 10, 20, 30, 40 and 50 ng ml^{-1} of B1 and G1 and 3, 6, 9, 12 and 15 ng ml^{-1} of AFB2 and AFG2 were run through *HPLC* system and calibration curves were calculated (Levin, 2012).

RESULTS AND DISCUSSION

Chemical analysis of feedstuff: Dry matter, ash and crude protein content of all samples, collected during December 2014, January 2015 and February 2015 were determined. Crude protein levels of concentrated feed samples produced for cow (dairy), cow (beef), sheep and goat (dairy), sheep and goat (meat), broiler stage 1, broiler stage 2, layers stage 1 and layer stage 2 were found as 10.98, 10.10, 11.12, 10.60, 19.66, 18.71, 16.47 and 13.45% respectively. Crude ash contents of the samples were also determined and ash levels were recorded as 21. 49, 19.16, 19.57, 21.17, 25.06, 19.42, 20.18 and 22.72% respectively for the samples mentioned above. For same samples, dry matter contents were found as 88.5, 89.84, 89.43, 88.12, 90.97 90.43 and 92.06% respectively. There was different nutrient composition of concentrated feeds in comparison with the standard of NRC (2002) values. Its mean that the factories in these areas added high amount of calcium carbonate. The lowness of protein due to high price of due to financial adverse effects of the interior war and there was no proper governmental authority on the factories and no periodic check to the factories producing animal feed in this area.

Molecular characterization: The fungi were isolated from a concentrated feed by culturing them on PDA agar and they were purified on selective media (AFPA) to identify the *A. flavus* and *A. parasiticus* according to their basic morphological characters. *A. flavus* produced yellow green colonies and they were predominantly biseriate or uniseriate with smooth to finely rough conidia. *A.parasiticus* had dark green colonies with a diameter of between 24-36 mm, was *predominantly uniseriate* with 20% biseriate and had rough conidia. The samples investigated here were mainly contaminated by *Aspergillus flavus* and this incidence was recorded regardless of temperature variation (15-20°C) and the remarkable level of the rainfall (over 400 mm/year) during sampling period which had not recorded in that area for over a decade. These results are in agreement with Abbas *et al.* (2004) who observed greater variation in aflatoxin formed by *A. flavus* different aflatoxin production capabilities of *A.flavus* strain would be influenced by different sources of strains and environmental conditions. relatively higher temperatures and drier conditions are known for the ability of infection by *A. flavus* but in this study the situation is not similar to the results observed in Nigeria (Atehnkeng *et al*., 2008). The study may be affected by the number of samples and short period of the collection of samples. Toxicogenic strains of *A. flavus* were more prevalent than non-toxinogenic strains through Northern part of Syria. The widespread occurrence of the fungus indicates the extent of pre-harvest infection; so, field management strategies stand out as an indispensable intervention strategy towards the fight against aflatoxin contamination of compound feed in exemplified region of Syria. Moreover, presence of these fungi may refer to the extent of pre-harvest infection because of lack of authority and consequently no strategies to control the system against contamination by aflatoxin in Syria after interior war started. The results are intersected with other studies in referring that a lot of strain of *A. flavus* isolated from corn and other widely varying materials have a high potential for aflatoxin production (Reddy *et al*., 2009a & 2009b).

Aflatoxin production on YES: All isolates were positive for either one or both gene and observed under UV light in YES media after 3 days of incubation at 28°C to test their aflatoxin production profiles. It was found that all the isolates were positive (*aflatoxinogenic*). That means all isolates had an aflatoxin production profile (Fig. 1). These findings were in paralel of the results of Konietzny and Greiner, (2003) who reported that the samples, they analyzed, had 50% aflatoxin positive (aflatoxinogenic) on YES agar under UV light. Due to fact that mycotoxins are important issues because of health and economic considerations, more sensitive and rapid techniques to detect them are desired. Strains were cultivated in Yeast Extract Sucrose (YES) medium and photographed on the third day of incubation at 28°C.

The genes controlling Aflatoxin production: All fungal isolates were examined weather they have the genes responsible for aflatoxin formation, and 6 of them were found to be positive for *aflD* gene which responsible for aflatoxin biosynthesis from averantin (Fig. 1).

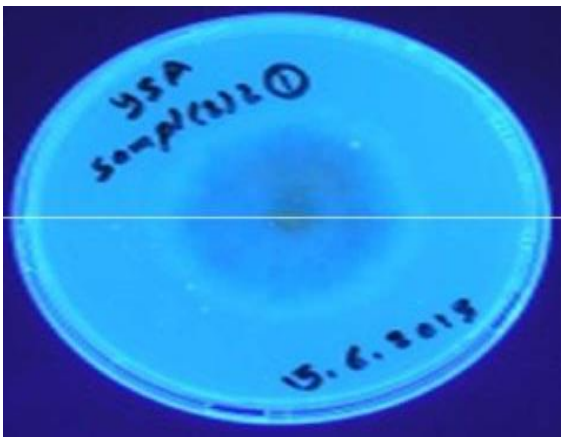


Fig. 1: *An exemplified culture of Colonies of a Aflatoxinogenic strains of Aspergillus grown in YES agar containing petri. visualised under ultra violet lightened. PCR amplification results of Aspergillus flavus run on 1% agarose Electrophoresis gel electrophoresis and photographed under 312nm UV light. of Aspergillus flavus DNA extracted in lab and isolated in AFPA; *Media M: 100 bp Marker (Favorgen, Taiwan); aflD and aflQ: 400 bp positive samples for aflD and 719 bp positive samples for aflQ.

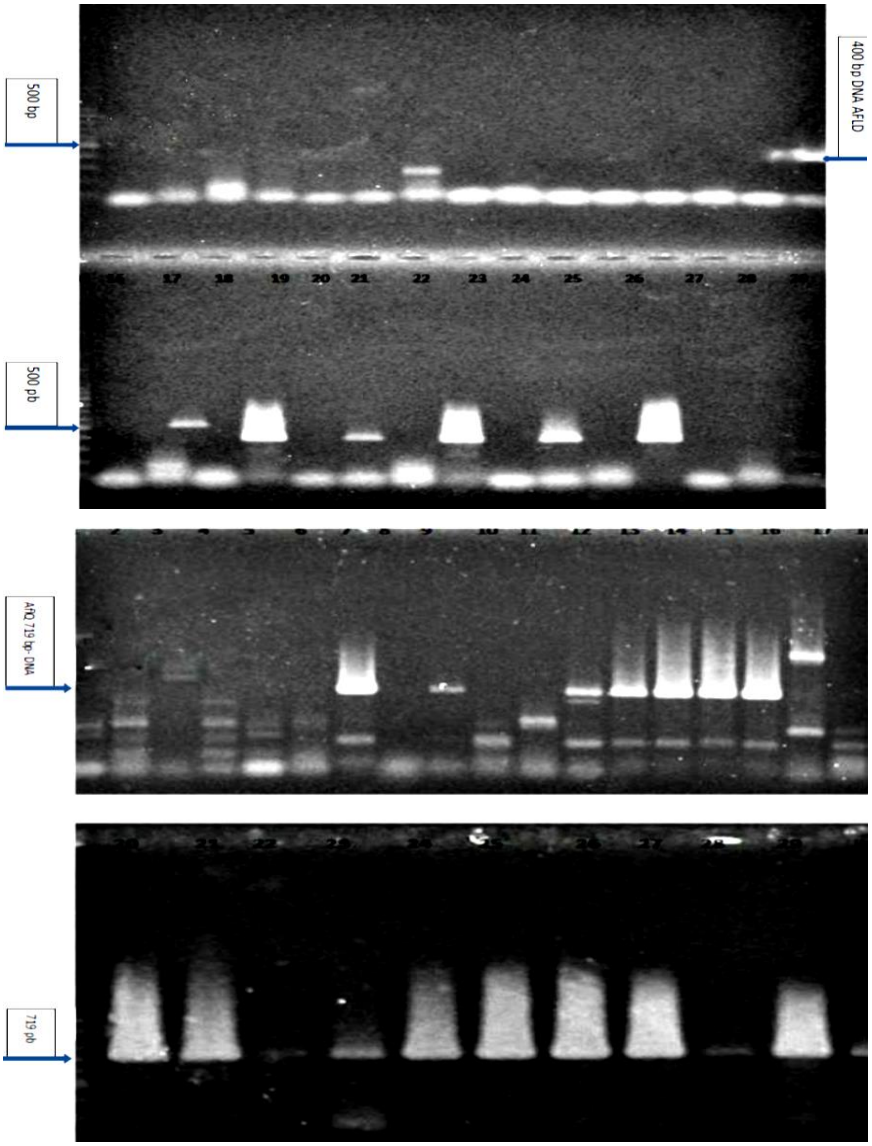


Fig. 2: PCR amplification results of Aspergillus flavus run on 1% agarose gel electrophoresis and photographed under 312nm UV light. M: 100 bp Marker (Favorgen, Taiwan); 400 bp positive samples for aflD and 719 bp positive samples for aflQ

Total of 18 positive samples for *aflQ* which responsible for biosynthesis o-methylstrigmatocystin to aflatoxin (Fig. 2) were found and expression of it had been reported as showing a high correlation of aflatoxinogenic ability.

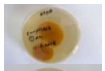
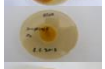
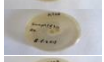
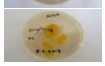
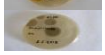
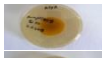
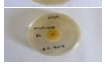
The *aflQ* gene was specifically chosen because it is considered to be the only gene involved in the final step of transforming o-methylsterigmatocystin *(OMST)* into aflatoxin, crucial step of aflatoxin pathway that seems to be unique to aflatoxigenic species (Levin, 2012; Scherm et al. 2005). As expected the presence of these two genes could not be correlated with aflatoxin producing ability.

Isolates were found 21. 30 (70%) positive and distinct colour characterization for selective media. All isolates were confirmed as *Aspergillus* section *flavi* by the bright orange colour of the colony reverse on *AFPA* media and remarked that all isolates in YES media under ultra violet lights had aflatoxin produced. It is mean that 100% of isolates had aflatoxinogenic characterization. There were 6 isolates were positive for *aflD (nor-1>).* That means these isolates have the step from aflatoxin biosynthesis series, which responsible for aflatoxin biosynthesis from averantin (Table 1).

We detected 18 samples are positive for *aflQ (ord-1)* (Table 1). These samples have a step from aflatoxin biosynthesis series, which responsible for transition of methylsterigmatocystin to aflatoxin. There was 6 (20%) common positive both *aflQ* and *aflD* observed them in concentrated feed from Northern Syria. In general, climatic condition in this area was not favorable for aflatoxin contamination. However, reduced rainfall and increased temperature during manufacturing can occur with associated levels of aflatoxin B1 in the excess legal limit (Piva and Pietri, 2006).

Measuring of Aflatoxin levels by *(HPLC-F):* Aflatoxin levels of all feed samples collected from Northren Syria during December 2014, January 2015 and February 2015, were also determined (Table 1). These samples were analyzed by *HPLC-F* to detected levels of B1. The levels ranged from high to standard levels in comparison with acceptable limit of aflatoxin B1 in animal feed material in Syria (20 ppm), (0.354 ppm, 8. 247 ppm, 4.45 ppm, 1,10 ppm, 0,134 ppm, 0,133 ppm, 0.360 ppm, 0.025 ppm, 18.13 ppm, 32.936 ppm, 10.326 ppm, 0.324 ppm, 9.977 ppm and 0.427 ppm). Same results were observed in Nigeria (Atehnkeng *et al*., 2008) but high temperature and drier condition are known infection by *Aspergillus flavus*, consequently rasing in aflatoxin B1 levels.

Table 1: Molecular characterization in AFPA media and the content of Aflatoxin B1 in concentrated feeds

Sample	Month/year	Aflatoxin B1/ppm	AFPA Agar	YES	Nor-1	Ord-1
Cow/dairy	December 2014	0.354	-	+	-	-
Cow/beef	December 2014	8.247	-	+	-	-
Broiler stage1	December 2014	1.10		+	-	-
Broiler stage 2	December 2014	0.134		+	+	-
Layers stage 2	December 2014	0.133		+	-	-
Layers stage 3	December 2014	0.360		+	+	+
Cow/dairy	January 2015	18.133		+	+	-
Cow/beef	January 2015	32.936	-	+	-	-
Sheep and goat/dairy	January 2015	10.346		+	+	-
Sheep and goat/meat	January 2015	0.324		+	+	+
Broiler stage1	January 2015	0.126		+	+	+
Broiler stage 2	January 2015	16.648		+	+	+
Cow/dairy	February 2015	15.75	-	+	-	-
Cow/beef	February 2015	19.367	-	+	-	-
Sheep and goat/dairy	February 2015	-	-	+	-	-
Sheep and goat/meat	February 2015	9.977		+	+	+
Broiler stage 1	February 2015	0.427		+	+	+
Broiler stage2	February 2015	-	-	+	-	-

(+):Aflatoxin-producing Aspergillus sp when grown on YES media; (-):Aflatoxin formation has not been determined on that samples; NOR 1(+): The sample of Aspergillus positive for Nor- 1 (AflQ); NOR 1(-): The sample of Aspergillus negative for Nor- 1 (AflQ); ORD 1(+): The sample of Aspergillus positive for Ord- 1 (AflD); ORD 1(-): The sample of Aspergillus negative for Ord- 1 (AflD).

Similar results were reported from India, where aflatoxin B1 levels exceeded the permissible Indian regulatory limit of 30 ppm in 21% of ground nut samples and 26% of maize samples collected from 11 different states. Therefore, it is so imported particularly for underdeveloped or developing countries (such as Syria) that Aflatoxin B1 levels had to be considered along with total aflatoxin before accepting or choosing a feed or feed ingredient for dietary use in animals (Bhat *et al.,* 1997; Bagheri and Mojtaba, 2014).

Conclusions: This study has displayed that *A. flavus* is the main fungal species infecting compound feed in Northern part of Syria and that toxicogenic strains are widespread. The influence of Aflatoxin on human population's in Syria over the past decade demonstrates a clear need to improve novel pathways to manage contamination of locally produced animal feed stuff. Given the extended nature of *toxicogenic* strains, any control strategy will have to include field management. Fungal infection of compound feed varies with households and this reflects different farm management practices, some of which adversely contribute to contamination of the feed value chain. The compound feed in Syria was contaminated mainly with *A. flavus* and *A. parasiticus* whose occurrence frequencies varied according to different months during rainy winter season. Feeds and their ingredients were all contaminated with Aflatoxin B1 and B2. Aflatoxin levels of majority of samples were over the safe limit of *FDA* (20 ppm).

The results of this study showed that 100% of the tested *A. flavus* strains produced aflatoxins, and most of the aflatoxinogenic. *A. flavus* strains showed a high capacity for aflatoxins production in compound feed. The high production of aflatoxins in animal feed in Northern part of Syria may have been due to lowness in laws and legislation which control in factories especially during interior war. They must develop primary storage structures by using various fungicides or some other chemicals to reduce *Aspergillus* and consequently toxin production in food grains under storage conditions.

They should follow system based on the Hazard Analysis and Critical Control Point *(HACCP)* to managing the risks associated with aflatoxin contamination.

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REFERENCES

Abbas H, Zablotowicz R and Locke M, 2004. Spatial variability of aspergillus flavus soil populations under different crops and corn grain colonization and aflatoxins. Can J Bot 82:1768-75.

Anastassiades M, SJ Lehotay, D Štajnbaher *et al*., 2003. "Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J Aoac Int 86:412-31.

AOAC, 2001. Association of official analytical chemists -official methods of analysis, 25th Edn. AOAC, Washington, DC pp:556.

Atehnkeng J, Ojiambo PS, Donner M, *et al*., 2008. Distribution and toxigenicity of aspergillus species isolated from maize kernels from three agro-ecological zones in Nigeria. Int J Food Mic 122:74-84.

Bagheri THM and Mojtaba B, 2014. Aflatoxin B1 in feed stuffs of dairy farms in shahrekord, Iran. J Nov App Sci 2014-3-11/1312-1316.

Bryden WI, 2007. Mycotoxins in the food chain: human health implications. Asia Pac J Clin Nutr 16 Suppl 1:95-101.

Bhat RV, Vasanthi S, Rao BS, *et al*., 1997. Aflatoxin B1 contamination in maize samples collected from different geographical regions of india multicentre study. Food Add Cont 14:151-6.

Hepperly P, Mignucci J and Sinclair J, 1981. The microorganisms of stored soybean seeds. soybean seed quality and standard establishment: proceedings of a conference for scientists of Asia. Agrarian Res Training Institute, Colombo, Sri Lanka.

Konietzny U and Greiner R, 2003. The application of pcr in the detection of mycotoxigenic fungi in foods. Brazil J Microbiol 34:283-300.

Levin RE, 2012. PCR detection of aflatoxin producing fungi and its limitations. Int J Food Microbiol 156-66.

Manonmani HK, Anand S, Chandrashekar A, *et al*., 2005. detection of aflatoxigenic fungi in selected food commodities by PCR. Process. Biochem 40:2859-64.

Markov K, J Pleadin, M Bevardi, *et al*., 2013. Natural occurrence of aflatoxin b 1, ochratoxin a and citrinin in croatian fermented meat products. Food Cont 34:312-7.

Meggs WJ, 2009. Epidemics of mold poisoning past and present. Tox Health 25:9-10

NRC, 2000. Nutrient requirements of beef cattle, 7th Revised Ed. Washington, DC, National Academy Press.

Naseem MN, Saleemi MK, Khan A, *et al*., 2018. Pathological effects of concurrent administration of aflatoxin B1 and fowl adenovirus-4 in broiler chicks. Microbial Pathog 121:47-154.

Pitt JI, Hocking AD, and Diane A, 2009. Fungi and food spoilage, Springer, New York 2009.

Piva G, Battilani P and Pietri A, 2006. Emerging issues in Southern Europe: Aflatoxins in Italy, Wageningen Academic Publishers: Wageningen, The Netherlands: pp:139-53.

Reddy K, Reddy C and Muralidharan K, 2009a. Detection of aspergillus spp. and aflatoxin B1 in rice in India. Food Microbiol 26:27-31.

Reddy K, Reddy CS, Kumar PN, *et al*., 2009b. Genetic variability of aflatoxin b1 producing aspergillus flavus strains isolated from discolored rice grains. World J Microbiol Biotech 25:33-9.

Rodrigues P, Venancio A, Kozakiewicz Z *et al*., 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of aspergillus section flavi isolated from portuguese almonds. Int J Food Microbiol 129:187-93.

Saleemi MK, Khan MZ and Khan A, 2012. Molecular identification of black Aspergilli isolated from poultry feeds by sequencing their ITS region. Pak Vet J 32:171-4.

Scherm B, Palomba M and Serra D, 2005. Detection of transcripts of the aflatoxin genes afld, aflo, and aflp by reverse transcription. Int J Food Microbiol 98:201-10.

Somashekar D, Rati ER and Anand S, 2004. Isolation, numeration and PCR characterization of aflatoxigenic fungi from food and feed samples in India. Food Microbiol 21:809-13.

Sweeney MJ, Pàmies P and Dobson AD, 2000. The use of reverse transcription-polymerase chain reaction (RT-PCR) for monitoring aflatoxin production in aspergillus parasiticus. Int J Food Mic 25:97-103.

Yu J, 2012. Current understanding on aflatoxin biosynthesis and future perspective in, pathway gene cluster in aspergillus parasiticus. Febs Letters 564.1:126-130. Reducing Aflatoxin Contamination. Toxins 4:1024-57.