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Recent Advances for Control, Counteraction and Amelioration of Potential Aflatoxins in Animal Feeds

N.K.S. Gowda, H.V.L.N. Swamy and P. Mahajan

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1. Introduction

Mycotoxins are the toxic products of fungal metabolism occurring in a wide variety of commodities like animal feeds and human food products. Mycotoxins on ingestion can cause health hazards both in livestock and human beings and hence there is a greater economic and public health implication. The severity of mycotoxin contamination is determined by environmental factors like excessive moisture in the field as well as in storage, hot and humid climate and insect infestation. Mycotoxin contamination of feed affects practically all livestock but greater information is available on dairy cattle, poultry, and swine. In these animals mycotoxins reduce production efficiency, impairs resistance to infection and compromise reproduction. Economic losses due to mycotoxicosis are derived directly from livestock morbidity, mortality and wastage of contaminated feed. On a global scale, it is estimated that around 25% of the world's crops are affected by mycotoxins annually and in addition to the above losses costs involved in monitoring the level of mycotoxins should also be considered. The recent mycotoxin surveys have indicated that the percent contamination is much higher than the perceived 25%. The mycotoxins that are of significance in animal feed are: Aflatoxins, Ochratoxins and Fusarial toxins (Fumonisin, Zearalenone, Trichothecenes including Deoxynivalenol and T-2 toxin).

1.1. Aflatoxins and biological action

The aflatoxins are highly toxic and carcinogenic compounds produced by *Aspergillus* fungi at an optimum temperature of 25-32^o C, moisture of greater than 12-16% and a relative humidity of 85%. Commonly affected feeds are maize, groundnut cake, cottonseed cake and copra cake and causes toxicity in poultry, cattle, sheep and swine. Animal consuming aflatoxin contaminated feed display poor performance, reduced immunity, liver damage, kid-

ney and intestinal haemorrhage and liver tumors. Among the afltoxins B₁ is more prevalent and toxigenic. This is metabolized to Aflatoxin M₁ in liver and is excreted in milk of dairy cattle and also as residue in egg / meat.

Epoxide derivative of aflatoxin B₁ binds with DNA and disrupts transcription and translation activities, thus initiating carcinogenesis. Oxidative nature of the toxic derivative releases free radicals and cause cell damage (Fig.1). Advancement in molecular techniques like microarray and PCR has helped to understand the precise mechanism of action of aflatoxin. Recent gene expression studies have shown that down regulation of mitochondrial carnitine palmitoyltransferase (CPT) system, down regulation of fatty acid metabolism pathway, up-regulation of cell proliferation pathway and down regulation of B cell activation are respectively responsible for decreased body weight gain, fatty liver / increased liver weight, carcinoma and lowered immunity in birds fed aflatoxin. Supplementation of curcumin through turmeric powder ameliorated most of the ill effects induced by aflatoxin. Adverse effects of aflatoxicosis are much severe when there is a concurrent contamination with other toxins like ochratoxin and T-2 toxin.

1.2. Limits of aflatoxin

The presence of Aflatoxin M₁ in food products meant for human consumption is not desirable and the residual concentration should not exceed 0.5 ppb as per FDA regulations. Such regulations are much more stringent in European Union where the level should not exceed 0.05ppb. Aflatoxin B₁ level of 20 ppb in the diet of dairy cattle is appropriate for reducing the risk of aflatoxin M₁ in milk. In many countries there are strict guidelines for maximum tolerable limits of aflatoxins, beyond which the commodity is unsafe and not accepted (Table 1).

Limits

Cattle 20 ppb, Broiler chicken 20 ppb, Finisher pig 200 ppb
 Beef cattle 300 ppb, Layer poultry 100 ppb
 India : 60 ppb (B₁) for groundnut cake, 120 ppb (B₁) for groundnut cake (export)
 UK & Spain : Complete feeds 10-20 ppb(B₁). Groundnut 50 ppb (B₁)
 Other feed ingredients 200 ppb (B₁)
 EEU : 500 ppb (B₁) for feed ingredients ; France : 300 ppb (B₁) for feed ingredients; Japan : 1000 ppb (B₁) for raw materials, 50 ppb (B₁) for complete feeds of cattle, 20 ppb (B₁) for complete feeds of pigs and poultry
 USA : 300 ppb (B₁) for cottonseed meal; 20 ppb (B₁) for other feed ingredients, milk for human consumption 0.5 ppb.
 Canada : 20 ppb (total aflatoxins) for livestock feeds
 South Africa : 10 ppb(total), Australia : 15 ppb (B₁) for groundnut

Table 1. Suggested limits for aflatoxin.

2. Control and counteraction of aflatoxins

2.1. Preventive measures

Aflatoxins affect mainly liver and kidney and are also carcinogenic and mutagenic (Fig 1). Therefore effective control and detoxification measures need to be undertaken. Toxin producing fungi may invade at pre-harvesting period, harvest-time, during post harvest handling and in storage. According to the site and time of infestation, the fungi can be divided into three groups: (a) Field fungi (b) Storage fungi (c) Advanced deterioration fungi. Field fungi are generally plant pathogenic fungi; namely *Fusarium*. The storage fungi are *Aspergillus* and *Penicillium*. The advanced deterioration fungi, normally do not infest intact grains but easily attack damaged grains and requires high moisture content, that include *Aspergillus clavatus*, *Aspergillus fumigatus*.

Prevention and effective plan for reducing fungal growth and toxin production is very important. The recommended practices include 1. Development of fungal resistant varieties of plants, 2. Suitable pre-harvest, harvest and post harvest techniques, 3. Store commodities at low temperature as for as possible, 4. Use fungicides and preservatives against fungal growth and 5. Control of insect damage in grain storage with approved insecticides.

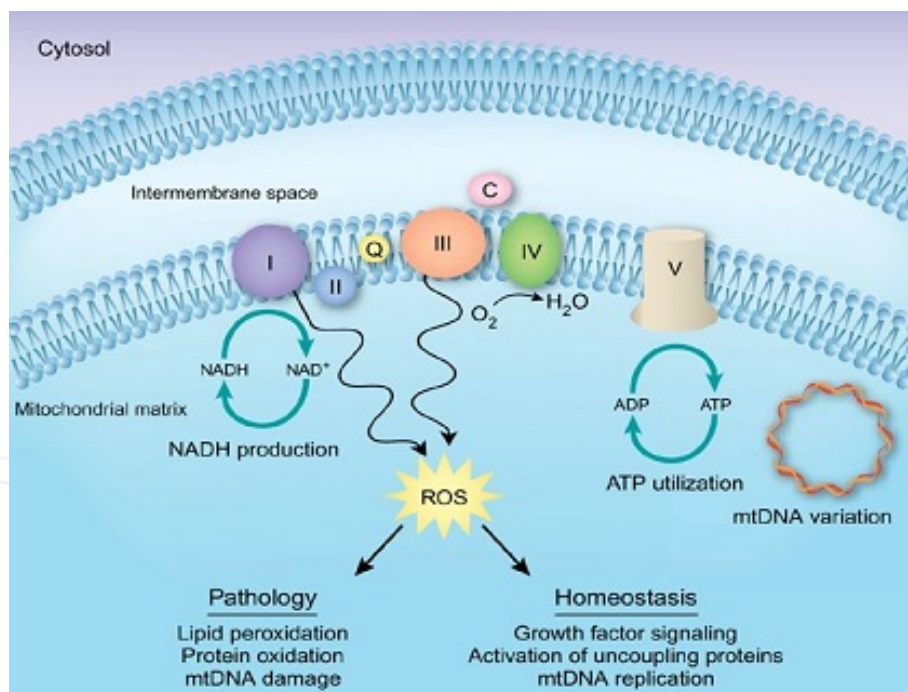


Figure 1. Mechanism of cell damage in mycotoxin toxicity.

(adopted from Joshua M Baughman and Vamsi K Mootha, 2006) [6]

The secondary prevention of fungal growth include limiting the growth of infested fungi by re-drying the product, removal of contaminated seeds. The tertiary measures could be to prevent the transfer of fungi and their health hazardous toxins into the food/feed and to the

environment. This include complete destruction of the contaminated product or diversion for fermentation to produce ethanol or detoxification / destruction of mycotoxins to the minimum level. Among the mycotoxins, aflatoxin is the most well-known and thoroughly studied and its prevention and control has been most successfully practiced in many countries.

2.2. Fungal growth inhibition

The inhibition of fungal growth can be achieved by physical, chemical and biological treatments. After the crop is harvested, drying and proper storage and suitable transportation of the commodities are of prime importance. Factors contribute to the growth of fungi and toxin production includes high moisture content, humidity, warm temperature (25-40 °C), insect infestation and grain damage.

2.2.1. Physical methods

- Drying seeds and commodities to the safe moisture level (< 9-11%).
- Maintenance of the container or store house at low temperature and humidity.
- Keep out insects and pests from the storage.
- Gamma-irradiation of large-scale commodities.
- Dilution of the contaminated feed with safe feed.

2.2.2. Chemical methods

- Use of fungicides (acetic acid, propionic acid, benzoic acid, citric acid and their sodium salts, copper sulfate): 0.2–0.4 % in feed.
- Use of fumigants – ammonia: 0.2-0.4%
- Addition of herbal extracts (garlic, onion, clove oil, turmeric powder, thyme) : 0.25-0.5%

2.2.3. Biological methods

Anti-fungal enzymes, chitinase and Beta -1,3 glucanase found in plant seeds, may act as defense against pathogenic fungi as chitin and glucan are major polymeric components of many fungal cell walls. Such polysaccharides in fungal cell wall could be enzymatically hydrolysed into smaller products resulting in killing of mycelia or spore of fungi. It is foreseen that seeds rich in such anti-fungal enzymes likely to resist the infestation of fungi. Use of non-toxicogenic biocompetitive *Aspergillus* strains to out-compete the toxigenic isolates has been found effective in reducing pre-harvest contamination with aflatoxin in peanut and cotton. However, the aflatoxin contamination process is so complex that a combination of approaches will be required to eliminate toxin production.

Application of non-toxicogenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* to soil in maize plots, favoured the reduction in colonization of toxigenic fungi in subsequent years. When the weather conditions were suitable for fungal growth and resulted in 65-80% de-

cline in aflatoxin production as compared to control. Inoculation of chitosan, *Bacillus subtilis* and *Trichoderma harzianum* to pre-harvest maize along with *Aspergillus flavus* inhibits aflatoxin production. Many anti-fungal metabolites (cyclic dipeptides, phenylactic acid, caproic acid, reuterin, lactic acid, acetic acid, fungicin) have been isolated from different cultures of lactic acid bacteria. Aflastin A, an anti-microbial compound produced by *Streptomyces* Spp., MRI 142 strain of bacteria is known to inhibit aflatoxin production by *Aspergillus parasiticus*. Iturin, an anti-fungal peptide produced by *Bacillus subtilis* had inhibitory effect on *Aspergillus parasiticus*.

2.2.4. Plant breeding, genetic engineering and microarray

Genetic modification of mold susceptible plants holds some promise in ensuring food safety. This involves increasing production of compounds like anti-fungal proteins, hydroxamic acids, and phenolics that reduce fungal contamination. This may be accomplished by introducing a novel gene to express the target compound, or enhancing the expression of such compounds by the existing genes, thereby capitalizing on the plant's own defense mechanisms. Enzymes that catalyze production of anti-fungals could be targeted for their expression and such an approach is being actively pursued by researchers. Enhanced expression of an alpha-amylase inhibitor in *Aspergillus* could result in reduced aflatoxin synthesis. Hybrid varieties of cereals with Bt (*Bacillus thuringiensis*) genes have shown reduced aflatoxin production, probably due to higher resistance of plants against pest and insects.

A cluster of genes are responsible for aflatoxin production through pathway-specific transcriptional regulator. A total of 20 genes in the aflatoxin biosynthetic cluster and 3 additional genes outside the aflatoxin biosynthetic cluster responsible for aflatoxin production have been identified. Identification of critical genes governing aflatoxin formation could lead to use of non-aflatoxigenic bio-competitive strains of *Aspergillus flavus* through use of gene disruption techniques. The advances in molecular biology could aid in early detection of mycotoxin production in food/feed material. DNA-chip with microarray system containing oligonucleotide primers that are homologues to genes of several fungal species responsible for the expression of mycotoxins can be employed to forecast the mycotoxin production in advance and accordingly critical anti-fungal strategies can be employed. Such PCR based molecular techniques are of value in assessing the potential for mycotoxin production. The time gap between expression of a set of genes and actual mycotoxin production is about 4-5 days. This early forecasting of extent of mycotoxin production will help in adopting immediate preventive anti-fungal measures.

2.3. Counteraction / Detoxification of aflatoxins

Aflatoxins in foods and feeds can be removed, inactivated or detoxified by physical, chemical and biological means. The treated products should be health safe from the chemicals and their essential nutritive value should not be deteriorated.

2.3.1. Physical methods

Physically, aflatoxin contaminated seeds can be removed by hand picking or photoelectric detecting machines, but this is labor intense and expensive. Heating and cooking under pressure can destroy nearly 70% aflatoxin. Dry roasting can reduce about 50-70% of aflatoxin and sunlight drying of aflatoxin contaminated feed could reduce the toxin level by more than 70%.

The addition of binding agents can reduce the bioavailability of these compounds in animals, and limit the presence of toxin residues in animal products. In case of aflatoxin B₁ (AFB₁), hydrated sodium calcium aluminosilicates (HSCAS) and phyllosilicates derived from natural zeolites have a high affinity, both *in vitro* and *in vivo*. Zeolites, which are hydrated aluminosilicates of alkaline cations are able to adsorb AFB₁. Bentonites have been shown to be effective for the adsorption of AFB₁. Other clays, such as kaolin, sepiolite and montmorillonite, bind AFB₁ but less effectively than HSCAS and bentonite. Activated charcoal has mixed results against AFB₁.

Although clays are effective against aflatoxins, caution should be exercised to make sure that their inclusion level is not too high and they are free from impurities such as dioxin. When the level of inclusion is very high, which is actually required for them to be effective, there are chances that these compounds can bind minerals and antibiotics like monensin. Some of the binders are not biodegradable and could pose environmental problem.

2.3.2. Chemical methods

A variety of chemical agents such as acids, bases (ammonia, caustic soda), oxidants (hydrogen peroxide, ozone, sodium hypochlorite), reducing agents (Bisulphites), chlorinated agents and formaldehyde have been used to degrade mycotoxins in contaminated feeds particularly aflatoxins. However, these techniques are not totally safe, are expensive and not well accepted by consumers.

2.3.3. Biological / microbiological methods

The biological decontamination of mycotoxins using yeast *Saccharomyces cerevisiae* and lactic acid bacteria has received much attention. Yeast and lactic acid bacterial cells are known to bind different toxins on the cell wall surface. This will be of immense value in reducing the mycotoxin hazards (Table 2), and effective binding strains of these microbes could eventually be used to minimize aflatoxin exposure and improving overall health in animals.

To tackle the high inclusion levels of clays, cell walls of specific yeasts were studied for their ability to bind aflatoxins. The wealth of data to date has shown that beta-glucans (esterified glucomannans), specific sugars present in the inner cell wall of yeast, can bind aflatoxins. The levels of inclusion of yeast-based binders are much lower than clay-based binders. About 500 gm of glucomannans from yeast cell-wall have the same adsorption capacity as 8

kg of clay. This binder reduces the AFM₁ content of milk by 58% in cows given a diet contaminated with AFB₁ at a concentration of 0.05% of dry mater.

Probiotic strain of *Lactobacillus acidophilus* CU028 has shown to bind aflatoxin. Probiotic fermented milk containing *Lactobacillus casei* and *Lactobacillus rhamnosus* strains alone or in combination with chlorophyllin exhibited protective effect against aflatoxin B₁- induced hepatic damage. Acid treated lactic acid bacteria were able to bind high dosage of aflatoxin in gut conditions.

2.3.4. Biotransformation

Dual cultivation of *Aspergillus niger*, *Mucor racemosus*, *Alternaria alternata*, *Rhizopus oryzae* and *Bacillus stearothermophilus* with toxigenic strain of *Aspergillus flavus* results in 70-80% degradation of aflatoxins. Certain microbes are also able to metabolize mycotoxins (*Corynebacterium rubrum*) in contaminated feed or to biotransform them (*Rhizopus*, *Trichosporon mycotoxinivorans*, *Rhodotorula rubra*, *Geotrichum fermentans*). However, these biological processes are generally slow and have a varied efficiency. Ruminants are considered to be relatively resistant to aflatoxins, due to biodegrading and biotransforming ability of rumen microbes compared to monogastric animals. This would be a great asset in biological detoxification of aflatoxins and with the help of genetic engineering techniques, benefits of this can be better realized.

Isolates	Number of aflatoxin B ₁ binding strains			
	Percentage of binding			
	<15	15-39	40-59	>60
Yeast				
<i>Saccharomyces cerevisiae</i>	1	8	3	3
<i>Condida krusei</i>	4	5	1	1
<i>Candida parapsilosis</i>				
<i>Trichosporon mucooides</i>	1	-	-	-
<i>Candida catenulanta</i>	1	-	-	-
Bacteria				
<i>Lactobacillus plantarum</i>	-	-	4	1
<i>Lactobacillus fermentum</i>	-	-	-	1
<i>Pediococcus acidilactici</i>	-	-	1	-

Table 2. Aflatoxin binding ability of different strains of yeast and bacteria.

2.4. Dietary manipulations

2.4.1. Hepatotropic nutrients and anti-oxidants

Various nutritional strategies have been employed to alleviate the adverse effects of aflatoxins. Addition of specific amino acids like methionine in excess of their requirement protect the chicks from growth depressing effects of AFB₁, possibly through an increased rate of detoxification by glutathione, a sulfur amino acid metabolite. Supplementation of phenyl alanine has shown to alleviate toxicity of ochratoxin. Addition of vegetable oil (safflower oil, olive oil) to aflatoxin contaminated feed improves the performance of chicks.

Aflatoxins cause toxicity through release of free radicals and lipid peroxidation. Hence, anti-oxidants could aid in the overall detoxification process in liver and hence may help in alleviation of aflatoxicosis. Butylated hydroxy toluene (BHT) is effective in preventing the adverse effects of AFB₁. Vitamin E and Selenium supplementation also has shown to overcome negative effects of aflatoxin. Of late, there is a growing interest in the use of phytochemicals (curcumin, flavonoids, resveratrol, Allixin, polyphenolics) as antioxidants in increasing the activity of antioxidant enzymes (SOD, catalase, glutathione peroxidase) and neutralizing the free radicals, thus, ameliorating the mycotoxin toxicity.

3. Conclusion

Aflatoxins are common in nature, hence minimizing the contamination is not an a easy task due to the interaction of fungus with environment and feed material. This involves constant attention during the entire process of grain harvest, storage, feed manufacturing and animal production. Most effective methods (physical, chemical, biological, biotechnological) to improve seed production, cultivation, harvest and storage need to be adopted. Use of binders and understanding their mechanism of action is the current concept and research areas in the use of microbes for decontamination and biotransformation of aflatoxins is gaining momentum. Biotechnological intervention in terms of developing transgenic fungal resistant crops and biological control using non-toxicogenic, competitive fungal species holds a better promise in managing the problem of aflatoxicosis. Advancement in molecular techniques using fungal oligonucleotide probes with PCR based microarray analysis would help in early forecasting / detection of potential aflatoxin production, suggesting for critical control strategies.

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binder. They are present in over 120 countries and operates on ACE Principle- providing solutions to the animal industry which are friendly to animals, consumer and environment

Author details

N.K.S. Gowda^{1*}, H.V.L.N. Swamy² and P. Mahajan²

*Address all correspondence to: nksgowda@rediffmail.com

1 National Institute of Animal Nutrition and Physiology, Bangalore, India

2 Alltech Biotechnology Private Ltd., Bangalore , India

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