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Comparative Evaluation of Different Techniques for Aflatoxin Detoxification in Poultry Feed and Its Effect on Broiler Performance

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1. Introduction

Aflatoxins (AF), the toxic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*, are a major concern in the poultry production. AF metabolites are stable and fairly resistant compounds to degradation (Dalvi, 1986; Park, 2002; Desphande, 2002; Lesson *et al*., 1995; Feuell, 1996). These metabolites are usually produced during the growth of the *Aspergillus flavus*, *Aspergillus parasitcus* and *Aspergillus nominus* on certain foods and feedstuffs under favourable conditions of moisture, temperature and aeration (Goto *et al*., 1997; Dutta and Das, 2001). Their toxicity depends on several factors including its concentration, the duration of exposure, the species, sex, age, and health status of animals (Jewers, 1990). Contamination of AF in feed causes aflatoxicosis in poultry that is characterised by reduced feed intake, decreased weight gain, poor feed utilization (Tedesco *et al*., 2004; Bailey *et al*., 2006; Shi *et al*., 2006, 2009), increased susceptibility to environmental and microbial stresses, and increased mortality (Leeson *et al*., 1995). AF can also cause productive deterioration which is associated with changes in biochemical and hematological parameters (Denli *et al*., 2004; Basmacioglu *et al*., 2005; Bintvihok and Kositcharoenkul, 2006), liver and kidney abnormalities, and impaired immunity, which is able to enhance susceptibility to some environmental and infectious agents (Ibrahim *et al*., 2000; Oguz *et al*., 2003). AF has been reported to have effect on metabolism in poultry by decreasing the activities of several enzymes that are important in the digestion of starch, proteins, lipids and nucleic acids. Consequently, the activities of serum glutamate pyruvatate transaminase, serum gluatamate oxaloacetate tranferase and γ-glutamyl transferase are increased, primarily indicating hepatic damage (Devegowda and Murthy, 2005). AF is also known to interfere with metabolism of vitamin D, iron and copper and can cause leg weakness (Khajarern and Khajarern, 1999). Severe economic losses have been reported in the poultry industry due to aflatoxicosis (Kubena *et al*., 1991, 1995). Ultimately, the transmission of AF and its metabolites from feed to animal edible tissues and products, such as liver and eggs, becomes a potential hazard for human health.

The occurrence of mycotoxin in nature is considered a global problem. However, in certain regions of the world, some of the mycotoxins are produced more commonly than others. Several *invitro* and *invivo* studies conducted in India, Pakistan, Egypt & South Africa suggested that AF are often present in substantial levels in mixed feed & ingredients (Devegowda and Murthy, 2005). Although, AF in feed and food is considered to be a major concern in warm and humid climatic regions of the world, however, caution must be exercised even in colder regions, when using feedstuffs imported from warm and humid countries.

With increasing knowledge and awareness of AF as a potent source of health hazards to both man and farm animals, producers, researchers and government organizations are making great effort to develop effective preventive management and decontamination technologies to minimise the toxic effects of AF content in foods and feedstuffs. In order to reduce the toxic and economic impact of mycotoxins, established regulations and legislative limits have been set for AF in poultry feed. Many countries follow a maximum permissible level of 20ppb for AF in poultry feed (CAST, 2003; FAO, 1995).

Appropriate pre and post-harvest contamination can be reduced by using appropriate agricultural practices. However, the contamination is often unavoidable and still remains a serious problem associated with many important agricultural commodities, which emphasizes the need for a suitable process to inactivate the toxin. Besides the preventive management, several approaches have been employed including physical (feed mill techniques, blending, extraction, irradiation, and heating), chemical (acids, bases, alkali treatments and oxidizing agents) biological treatments (certain species of fungi and bacteria) and solvent extraction to detoxify AF in contaminated feeds and feedstuffs (Coker *et al*., 1986; Piva *et al*., 1995; Parlat *et al.,* 1999). Since the beginning of 1990s, the adsorbent-based studies have also been reported to be effective in removing AF from contaminated feed and minimise the toxicity of AF in poultry (Ibrahim *et al.,* 2000). Among several adsorbents commercially available in the market, Zeolites (Miazzo *et al.,* 2000), bentonites (Rosa *et al.,* 2001, Pasha et al, 2007, 2008) and clinoptilolite (CLI), (Oguz and Kurtoglu, 2000; Oguz *et al.,* 2000 a, b), were preferred because of their high binding capacities for AF and their reducing effect on AF-absorption from the gastrointestinal tract.

All these methods cannot be used in practical feed manufacturing, because of the limitation of the nutrients decomposition, non availability of commercial methods and their residual effects. The increasing number of reports on detoxification of AF in poultry feed using different techniques has given rise to a demand for practical and economical detoxification procedures. Some of the physical treatments are reported to be relatively costly and may also remove or destroy essential nutrients in feed. Whereas, chemical methods are considered to be time consuming, expensive as they mostly require suitable reaction facilities, and are reported to have deteriorating residual effects on animal health (Coker, 1979; Coker *et al*., 1985). Certain legal implications are also associated with the use of different detoxifying methods. For example, European community (EC) is in favour of use of physical decontamination processes and sorting procedures. However, neither the use of chemical decontamination processes, nor the mixing of batches with the aim of decreasing the level of contamination below the maximum tolerable level is legal within the European Union (Avantaggiato *et al*, 2005). Although several mycotoxin detoxitoxifying or adsorbing techniques have been assessed independently however, limited information is available on the comparison of different techniques. To further understand the mechanisms of aflatoxin and detoxification of poultry feed by heat treatment (extrusion) and added adsorbents

(Sodium bentonite or Mycofix®Plus), a study was conducted to compare different detoxification techniques and to further investigate its effect on broiler performance.

2. Materials and methods

2.1 Birds and diet

Two hundred day-old commercial broiler chicks were randomly distributed to 5 dietary treatments with 4 replicates of 10 chicks each. During the first 21 days, all birds were fed on diet 1 which was the basal starter ration without any aflatoxin contamination (AF) and detoxifying treatment (DT). The ingredient composition of the basal diet is presented in Table 1. Experimental diets were prepared by replacing maize with contaminated maize having 70 ppb AF (Treatment 2) and were subjected to different DT (Treatment 3 to 5).

Ingredient	Starter (%)	Grower (%)
Maize	50.70	60.00
Rice tips	10.00	10.00
Corn gluten meal 60 %	5.00	3.00
Soybean meal	10.00	6.00
Guar meal	5.00	5.00
Cotton seed meal	7.00	7.00
Rape seed meal	7.00	7.00
Fish meal	3.00	
Molasses	1.00	
Di-calcium Phosphate	1.00	1.00
Lysine	0.30	0.35
Calculated nutrient composition		
M E (kcal/kg)	3000	3100
Crude protein	22.00	19.00
Crude fiber	4.53	4.48
Lysine	1.08	0.93
Methionine	0.49	0.45
Cystine	0.27	0.27
Met+Cys	0.88	0.80
Linoliec acid	1.21	1.36
Calcium	1.00	0.92
Phosphorous total	0.75	0.69
Phosphorus available	0.44	0.41

Table 1. Ingredient and nutrient composition of basal starter and grower diet of broilers.

On day 21, birds were fed on one of five experimental diets. Experimental diets were fed from day 21 to day 42 of the trial. Feed and water was available on *ad libitum* basis. All the birds were vaccinated against Newcastle Disease (N.D.), with Lasota strain eye droppings at day 7 and with oil based vaccine (intra muscular) at day 21 of the experiment.

2.2 Experimental design

The experimental design consists of five dietary treatments; 1 (0 ppb AF & no DT); 2 (70 ppb AF & no DT); 3 (70 ppb AF & DT by Extrusion); 4 (70 ppb & DT by Mycofix®Plus); 5 (70 ppb & DT by Sodium bentonite).

2.3 Aflatoxin production and analysis

Aflatoxins were produced by the inoculation of fungus on corn as described by (Lillehoj, *et al*., 1974) with some amendments. Fermentation was carried out in 1-liter Erlenmeyer flasks containing 50g of whole corn kernels. 25 ml distilled water was added to the corn (50 g) in the Erlenmeyer flasks, and the mixture was allowed to stand for 2 hrs with frequent shaking. The flasks were tightly plugged with cotton and autoclaved at 121^{o}C at 15 psi for 15 min and cooled at room temperature. They were then inoculated with 3ml spore suspension in a sterile environment, placed on an orbital shaker at 200rpm and incubated at 28 °C. At 24 and 48hr, sterile water (3-5ml) was added in the flask, quantity of water adjusted in a manner that individual kernels do not adhere with each other.

If the corn did pack in clumps, the material was loosened by vigorous shaking, and if required, clumps were smashed with the help of a sterile rod within sterile environment to make sure that individual kernel should be kept free from others. On 7-8d the flasks were again autoclaved at 121^{o}C at 15 psi for 15 min, and placed in a hot oven at 60 ^{o}C for 24hr till all the moisture was removed.

The AF containing corn Kernels were grinded to powdered form and was quantitatively evaluated by direct competitive enzyme link immuno-sorbent assay (ELISA) described by Barabolak (1977) using (RIDASCREEN® FAST Aflatoxin Total) kit and mixed in feed according to the calculation to get the desirable level of aflatoxin (70 ppb) in the feed. The prepared experimental diets were analysed again using ELISA technique to confirm the AF levels.

2.4 Methods used for detoxification of AF

2.4.1 By extrusion

The AF containing corn was passed through the extruder, following the procedure described by Grehainge *et al,* 1983. The corn in the experimental starter and grower diets was replaced by extruded corn (Treatment 3).

2.4.2 By Mycofix

A commercially available Mycofix® plus was added in the experimental diet at the recommended dose rate of 0.5 Kg per ton of feed (Treatment 4).

2.4.3 By Sodium Bentonite (SB)

The source and composition of SB used was same as described previously (Pasha et al, 2007). The supplemental SB was added in the experimental diet at 1% of the feed (Treatment 5).

2.5 Sampling

Body weights and feed intake per pen was recorded weekly and mortality was recorded as it occurred. At day 28, 35 and 42 of bird's age, five birds per replicate were randomly selected for estimation of antibody titers against Newcastle disease (ND). The blood samples (3 ml) were collected from wing vein. The blood serum was separated and analysed by Haemagglutination inhibition (HI) method described by Sever (1962). After blood collection, birds were humanely killed and bursa of Fabricius was removed and weighed.

3. Statistical analysis

The results (pen means) were subjected to one–way ANOVA as a complete randomized design (CRD) using Genstat 11 for window. Treatment means were compared by the Tukey's test and statistical significance was accepted at $P < 0.05$

4. Results and discussion

4.1 Growth performance

The body weight gain, feed intake and FCR values showed no differences between different treatments (Tables 2). This lack of difference in performance between treatments could either be due to lower level of AF (70ppb) or shorter administrative period (day 21 to 42 of age). Similarly no change in production parameters have been reported previously in several studies (Ouz *et al.,* 2000b, 2003; Ortatatli *et al.,* 2005; Magnoli *et al.,* 2008a,b, 2011) when birds were fed diets low in toxin (50 to 100 ppb AF) for a period of 46 days.

The results from the present study are not in agreement with other studies where significantly reduced body weights were observed when birds were exposed to higher dietary AF (400 and 600 ppb AF). The depression in growth upon feeding AF was attributed to reduced protein and energy utilization (Smith and Hamilton, 1970; Lanza *et al.* 1980; Doerr *et al.,* 1983; Dalvi and Ademoyero, 1984; Verma *et al.,* 2002) which impaired nutrient absorption and reduced pancreatic digestive enzyme production (Osborne and Hamilton, 1981) and consequently reduced appetite (Sharline *et al*, 1980). Similarly, significant depressions in body weight gain were also recorded in broilers given diets containing 1 and 2 mg/kg of AF (1000 to 2000 ppb) at 4 and 7 weeks of age. Several studies have also shown that dietary AF adversely affected the growth of broilers in a dose-dependent manner (Johri and Sadagopan, 1989; Espada *et al.,* 1992; Beura *et al.,* 1993). A similar reductions in weight gain were also observed in broilers at dietary AF contents of 0.75 mg/kg (750 ppb) and above and these depression in body weight in toxin fed groups were reported to be dose dependent (Reddy *et al.,* 1984).

Treatment	DESCRIPTION	Weight Gain (g)	Feed Intake (g)	FCR
1	(0 ppb AF & no DT)	1566	3221	2.05
2.	(70 ppb AF & no DT)	1527	3215	2.10
3	70 ppb AF + DT by extrusion	1533	3249	2.11
4	70 ppb AF + DT with Mycofix®Plus	1544	3233	2.09
5	70 ppb AF + DT with Sodium bentonite	1564	3244	2.07
	SED	24.94	24.51	0.048
	P-value	0.444	0.608	1.00

Means within a column were not different ($P < 0.05$). Tukeys T test was used for means separation; SED – standard error of the difference.

Table 2. Weight gain, feed intake, and FCR of birds fed different experimental diets.

In contrast to our results, reduced feed intake and poor feed efficiency in broilers has also been reported in birds fed diets containing AF at 2, 4 and 6 weeks of age when level of dietary AF was higher than 100 ppb (Sharline *et al*. 1980; Huff and Doerr, 1981; Nandkumar *et al*. 1984; Rajasekhar Reddy *et al.* 1982; Johri and Majmudar, 1990; Verma *et al.,* 2004). These authors have suggested that the reduced appetite during aflatoxicosis could be due to impaired liver metabolism caused by the liver damage. The reason for these differences in performance compared to present study could be due to different type, age and dose of AF. It is likely that juvenile birds may respond differently to the same dose of AF in diet as their physiological needs and capacity to absorb is higher compared to older birds.

It is suggested that extrusion-cooking is an efficient process used for eliminating some of the naturally occurring food toxins. The process involves high temperature (up to 250°C), short time (usually 1-2 min), high pressures (up to 25 mPa) and low water contents (below 30) and has been used to eliminate some of the toxins in food and feed ingredients (Harper, 1989; Fast, 1991; Kohlwey et al. 1995;). It has also been used as a kind of bioreactor to decontaminate AF. In spite the fact that feed moisture, barrel temperature and the die diameter are identified as different variables that can influence AF reduction during extrusion cooking, a reduction in total AF content (up to 84%) has been reported when artificially contaminated peanut meal was detoxified using extrusion cooking (Grehaigne *et al*., 1983; Cheftel, 1986, 1989). Similarly, commercial adsorbents are also reported to bind the aflatoxins in feed and prevent its absorption in animal gastrointestinal tract (Ramos and Hernandez, 1997). Numerous studies have shown the effectiveness of these agents to bind aflatoxins in vitro (Huff *et al.,* 1992; Diaz *et al.,* 2002). Mycofix, is one of the adsorbent that can be added in poultry feed and is claimed to neutralize moderate levels of aflatoxin (up to 2500-3500 ppb) in poultry feed. Mycofix deactivates aflatoxin with its polar functional group, due to AF fixation to adsorbing components in Mycofix, with stable binding capacity. Adsorption starts in the oral cavity during salivation and continues in stomach and gut. The fixed mycotoxin being unable to enter the blood and subsequently excreted in faeces after 98% adsorption of AF by Mycofix (Biomin®, 2000). Similarly, incorporation of SB in poultry diet is another proved adsorbent, to have high AF binding capacities both in vitro (Magnoli *et al.,* 2008a) and in vivo (Rosa *et al.,* 2001; Magnoli *et al.,* 2008b). Bentonites are basically clays with strong colloidal properties that absorb water rapidly, which results in swelling and a manifold increase in volume, giving rise to a thixotropic, gelatinous substance (Bailey *et al,* 1998). Bentonites are composed of hydrated aluminosilicates of sodium (Na), potassium (K), calcium (Ca), and occasionally iron, magnesium, zinc, nickel, etc. They have a high negative charge and are balanced by cations such as Mg, K, and Na, therefore, they do not react with food/feed ingredients and act as inert material due to their neutral pH or slightly alkaline nature. However, the adsorption ability of these clays varies from one geological deposit to another.

In the current study, neither extrusion nor any of the absorbent (Mycofix®Plus and SB) resulted in any significant improvement in birds performance. The reason for lack of significant effects for DT methods used could probably be due to the performance of the birds on the AF containing diets (treatment 2). If the diet containing 70ppb AF (Treatment 2) had negatively influenced performance, it would be expected that DT methods used would restore or improve production. Therefore, this result does not imply that the DT methods used are not effective but rather indicate that birds exposed to higher levels of AF in diets are more likely to be benefited from the detoxified feed.

4.2 Antibody titre

The means of antibody titre (HA) against Newcastle disease (ND) showed no difference ($P>0.05$) between treatments when analysed at 21, 28, 35 and 42 day of the trial (Table 3).

The presence of AF in the feed is reported to decrease vaccinal immunity and may therefore lead to the occurrence of disease even in properly vaccinated flocks (Lesson *et al*., 1995). Aflatoxins have been associated to have immunosuppressive effect due to direct inhibition of protein synthesis, including those with specific functions such as immunoglobulins IgG, IgA, inhibition of migration of macrophages, interferance with the haemolytic activity of

complement, reduction in the number of lymphocytes through its toxic effect on the Bursa of Fabricius and impairment of cytokines formation by lymphocytes (Tung *et al.,* 1975; Creppy *et al.,* 1979, Devegowda and Murthy, 2005). In present study, no difference (P>0.05) in HA titres was observed when treatment with AF (treatment 2) was compared with all other treatment groups suggests that birds exposed to 70ppb AF in diet do not show any signs of immunosuppression. Similarly, Gabal and Azzam, (1998) suggested that prolonged administration of AF at the low levels do not markedly change the hematological and serological parameters of broiler chickens, but may cause relevant lesions in liver and renal tissues. Moreover, the metabolism of broilers seems to be more adapted to high concentrations of aflatoxin in the feed when administered from 21 to 42 d of age, when compared with data reported from similar experiments conducted with broilers aging 1 to 21 d and with other species such as turkey poults (Pier *et al.,* 1979; Campbell *et al.,* 1988; Gabal and Azzam, 1998).

Treatment	Description	HA		
		Day 28	Day 35	Day 42
1	0 ppb AF & no DT	14.9	257.6	184.0
2	70 ppb AF & no DT	13.7	222.9	138.9
3	70 ppb AF + DT by extrusion	17.1	268.9	168.9
4	70 ppb AF + DT with Mycofix®Plus	13.9	237.2	168.9
5	70 ppb AF + DT with Sodium bentonite	16.5	245.3	184.4
	SED	4.65	23.6	17.65
	P-Value	0.924	0.396	0.143

Means within a column were not different (P < 0.05). Tukey's test was used for means separation; SED – standard error of the difference.

Table 3. Effects of experimental diets on haemagglutination titres (HA) against ND at day 28, 35 and 42 of the trial.

In contrast to our study dietary AF has been reported to cause vaccine failure as indicated by significantly reduced (P<0.05) antibody titres against Newcastle disease vaccine when birds were fed diets containing 2000 to 3000 ppb AF (Rathore *et al.,* 1987; Mangat *et al.,* 1988; Viridi *et al.,* 1989; Ghosh *et al.,* 1990; Bakshi, 1991; Mohiuddin and Reddy, 1993; Sharma, 1993; Mohiudin, 1993). Similarly, Azzam and Gabal (1998) reported that even low levels of dietary AF (200 ppb) can cause reduction in antibody titers to vaccines for Newcastle disease, infectious bronchitis, and infectious bursal disease in layers, when fed for a longer period (40 weeks). This difference in response to HA titres results could be attributed to the higher inclusion levels of dietary AF (200 to 3000 ppb) used in these studies.

The use of feed adsorbents is considered the most promising and economical approach for reducing mycotoxicosis in animals. The beneficial effect of Mycofix® has been reported to ameliorate the negative effect of AF on IBDV antibody titres and the effects are attributed to the presence of phytogenic substances, a hepatoprotective flavolignins (silymarin) in Mycofix, which prevents toxins from entering the liver cell membranes, and as it contains the terpenoid complexes, which reduce inflammations and protect the mucous membranes (Biomin®, 2000). Similarly, Ibrahim et al., (2000) reported that SB is also effective in ameliorating the suppressive effect of AF on the HI-titer in chicks vaccinated against Newcastle disease and the best result was obtained when SB was added at a rate of 0.4% of feed to the AF-containing diets. This effect was attributed to the role of SB as a sequestering

agent against AF present in the diet through reducing its bioavailability in the gastrointestinal tract (Araba and Wyatt, 1991). However, in the present study, no differences (P>0.05) in ELISA titres were observed when birds fed AF diet (Treatment 2) were compared with all other treatment groups. This result further support our growth performance results and indicates that diets low in AF (70 ppb) do not depress broiler growth and vaccinal immunity.

4.3 Bursal body weight ratio (BBR)

The sensitivity of the immune system to mycotoxin-induced immunosuppression arises from the vulnerability of the continually proliferating and differentiating cells that participate in immune mediated activities and regulate the complex communication network between cellular and humoral components. AF are reported to inhibits the histological development and functional maturation of lymphoid organs (Celik *et al.*, 2000). Morphological evidence to explain the immunosuppressive effects of AF (2500 ppb) was documented by Celik *et al.* (2000) in broiler chickens after 21 days of feeding and the major signs were reduction in the weights of lymphoid organs including bursa of Fabricius, spleen and thymus. Similarly, Verma *et al.*, (2004) reported a significant decrease in the relative weight of the bursa of Fabricius when birds were exposed to diets having 2000 ppb AF. Similar reduction in BBR and moderate histopathological changes have been reported in broilers (Giambrone *et al.*, 1985; Marquez and Hernandez, 1995), laying hens (Dafalla *et al.*, 1987), ducks (Sell *et al.*, 1998; Khajarern and Khajarern, 1999) and wild turkeys (Quist *et al.*, 2000) when birds were fed diets having various levels of AF (100 to 500 ppb). In addition, vacuolation of liver cells and cellular depletion in the follicle medulla of the bursa Fabricii has been reported to be produced as an indication of aflatoxicosis by feeding lower levels of AF (100 ppb) over a long-term period of 42 days (Espada *et al.*, 1992). In contrast, the present study indicated no significant difference (P>0.05) in BBR between different treatment groups (Table 4). However, it cannot be concluded from the present investigation whether 70 ppb AF level in broiler diet can cause aflatoxicosis in broilers, as no significant difference (P>0.05) was observed when different response parameters tested were compared to the those of AF contaminated diet. This difference in results probably could be due to differences in age or genetic strain of birds, nutritional status, and source of mycotoxins, exposure time, vaccination schedule, serologic technique and management practices used in these studies.

Treatment	Description		BBR
1	0 ppb AF & no DT		1.69
2	70 ppb AF & no DT		1.54
3	70 ppb AF + DT by extrusion		1.77
4	70 ppb AF + DT with Mycofix®Plus		1.60
5	70 ppb AF + DT with Sodium bentonite		1.70
		SED	0.77
		P-value	0.998

Means within a column were not different (P < 0.05). Tukeys T test was used for means separation; SED – standard error of the difference..

Table 4. Effect of experimental diets on the average mean bursal body weight ratio (BBR) of birds at 42 days of age.

5. Conclusion

The manifestation and magnitude of a AF related response depends upon dose of AF, time period that the determined dose is exposed to the animal and interactions (such as age of animal, nutritional status at the time of AF exposure, presence of multiple mycotoxins in the diets etc). Prevention and control of AF in the poultry production chain requires the knowledge and consideration of all factors influencing mycotoxin formation in the field and during the storage of feedstuffs. The results from the current study demonstrated that growth performance and immune response was not depressed when broilers aged 21 to 42 days were exposed to diets containing 70ppb AF. However, methods of DT compared did not result in any significant improvement (P>0.05) in any of the response parameter. Further studies are recommended to evaluate the efficacy of the detoxifying agents by using a factorial designs that include a non-contaminated diet and a contaminated diet, both with and without DT.

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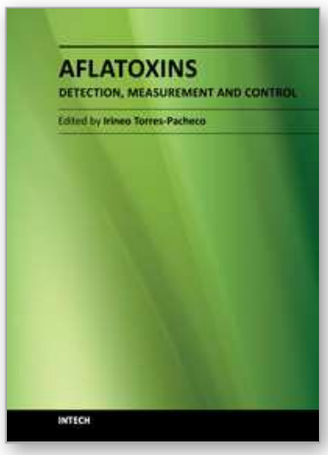

Aflatoxins - Detection, Measurement and Control

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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical prespective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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