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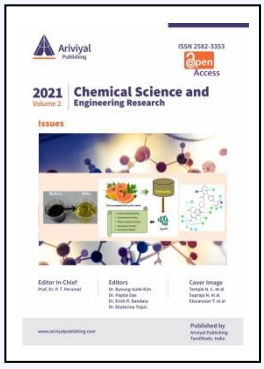

Aflatoxin Types, Permissible level, Factors Responsible for Aflatoxin Contamination, Determination and Detoxification Methods in Animal Feed

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Abstract: A group of toxins known as Aflatoxin is originated from two fungal species Aspergillus flavus and *Aspergillus parasiticus*. These important toxins in animal feed are classified into B1, B2, G1, and G2. The most toxic and abundant form of aflatoxin is B1 however, aflatoxin M1 is abundant in cow milk, a hydroxylated form of aflatoxin B. United State Food Drug Authority introduced highest concentration/accumulation of aflatoxin in animal feed. The permissible limit is 20µg/Kg in animal feed. Aflatoxins contamination in milk, milk products, and child food are restricted to zero. Countries have set up exceptional standards for aflatoxins degrees in domesticated animal feed. Factors such as physical, chemical, biological and nutritional are responsible for the growth of Aspergillus species and ultimately causes aflatoxin production. Different chromatographic methods like TLC, ELISA and HPLC are used for the detection of aflatoxins in animal feed. This review covers the information regarding aflatoxins types, permissible levels, detection and detoxification ways.

Keywords: Hydroxylated; TLC; HPLC; ELISA; Aflatoxins; Feed

1. Introduction

Aflatoxins are carcinogenic outgrowth of fungal species Aspergillus flavus and *Aspergillus parasiticus*.[1-53] They are commonly known types of mycotoxins (Zahra et al., 2019).[52] According to Food and Agricultural Organization, 25% of the world's crop is being destroyed by mycotoxins, mainly aflatoxins (Vincelli et al., 1995).[47] Peanuts, cereals maize, grains, and animal fodder are the agricultural foodstuff in which these two Aspergillus species are usually detected (Bennett et al. 1987).[6] Mortality, lower growth rates, immune system suppression, and loss in feed productiveness are the deleterious reactions of aflatoxins (Vincelli et al., 1995).[47] Aflatoxins are reviewed as a stable class of mycotoxins found in the food and are affected by numerous parameters such as temperature, protein presence, period of heat treatment, pH, and presence of organisms that assist in initiating the process (Scudamore, 1998).[40] In 1997, Food and Agricultural Organization declared the most toxic form of aflatoxins is Aflatoxin B_1 (AFB1). Aflatoxins are the ubiquitous cancerous natural secondary metabolites derived from fungal cells that cannot cultivate under unfavourable surroundings which can be climatic or befitting hygienic storage habitat (Curto et al., 2004).[11] Aflatoxins have low molecular weight and are discovered in 77 countries (F.A.O., 2004).[20] Acetonitrile water, methanol-water, or simply water can be used as chemicals to detect aflatoxins (Diaz et al., 2012).[15] Due to toxicity and carcinogenicity of aflatoxins, chiefly aflatoxin B1 release lesions in the liver of innumerable animals like rats and generate a 15ppb level of hepatic carcinoma (Butler, 1973).[9] The optimum conditions under which aflatoxins growth is observed are temperature 33 degrees Celsius and 0.99 a_w water activity (Ominski et al., 1994).[54] Many studies demonstrated that during checkpoints of cell cycle G_1, G_2, and M phase, in vitro cells respond to Aflatoxin B1 and as a result cause DNA degeneration. Oxidative stress is also occurred by AFB1, which further destroys cells, DNA, lipid, proteins and leads to tumorigenesis (Marin and Ionelia, 2012).[55] Aflatoxins triggers mutation in the tumor suppressor protein gene p53 which ultimately leads to a missense mutation (Dhanasekaran et al., 2011).[14] Different countries have adopted different aflatoxin permissible levels in animal feed (Zahra et al., 2019).[52]

2. History

In the early 1960s, a plaque outspread in turkeys, chickens, and ducks causing their death known as "Turkey X disease". It was further discovered that 5000 pheasants and partridges and 1400 ducklings died due to the outburst of aflatoxins in the farms. After that other

animals including pigs and calves also expired because of aflatoxin's upsurge (Loose more and Harding, 1961).[30] It was studied that agents responsible for the Turkey X disease are the microscopic aflatoxins present in peanut meal which animals feed and as a consequence testing initiate. Aspergillus flavus was found in the meal which is responsible for liver toxicity (Wogan, 2012).[49] Aflatoxins are released off in milk, serum, urine, and blood (De Cassia Romero et al., 2010).[12] The first country to conduct an aflatoxins study and its direct relation to the liver was Uganda (Pitt et al., 1998).[56]

3. Types of Aflatoxins

Aflatoxins are furanocoumarin molecules derived through the polyketide pathway (Bennett et al. 1987).[6] B_1, B_2, M_1, M_2, G_1, and G_2 are the six different kinds of important aflatoxins out of a total of 18 aflatoxins (Dors et al., 2011).[18] They have molecular differences such as lactone ring is present in G_1 and G_2 while cyclopentane ring is present in group B_1 and B_2 (Gourama and Bullerman, 1995).[22] Blue fluorescence is shown by B group Aflatoxins while yellow-green fluorescence is exhibited by G group Aflatoxins under UV light. The most common aflatoxin is AFB_1 in the world (Hussein and Brasel, 2001).[26] Aflatoxins M_1 and M_2 are the hydroxylated forms of aflatoxins B_1 and B_2 respectively. They are associated with cow milk upon insertion of aflatoxins B_1 and B_2 aflatoxins contaminated feed. Aflatoxins M_1 and M_2 remain stable during the milking process, once formed from aflatoxins B_1 and B_2 (Stroka and Anklam, 2002).[45] Aflatoxin B_1 metabolism takes place in the liver and is accomplished by combined function monooxygenases belonging to the cytochrome P_{450} superfamily of enzymes (Guengerich et al, 1998).[23] It was shown by thin-layer chromatography on silicic acid that a toxic factor, milk toxin having R_f value less than aflatoxin B_1 has a blue-violet fluorescence. The chromatographic experiment conducted demonstrates that an extract of an A. flavus culture on crushed peanut comprised a constituent identical to milk toxin (De Iongh et al., 1964).[13] This same material was also found in the liver, kidney, and urine of sheep that had been killed within two hours after administrating a single dose of mixed B_1, B_2, G_1, and G_2 and proposed a name aflatoxin M for milk toxin (Allcroft et al., 1966).[3] Repeating the experiment of Allcroft, Holzapfel segregated aflatoxin M_1 from the urine of sheep within 48 hours after injecting mixed aflatoxins and then prosper in dividing it into two components on paper chromatography, one component aflatoxin M_1 with blue fluorescence and other component M_2 with violet fluorescence. Aflatoxins B_1, B_2, G_1, and G_2 were segregated from each other by chromatography on silicic acid while aflatoxin M_1 and M_2 were segregated by chromatography on paper. Holzapfel also extracted aflatoxins M_1 and M_2 from moldy peanuts. They summarized that 4-hydroaflatoxin B_1 is aflatoxin M_1 and 4- hydroxyaflatoxin B_2 is aflatoxin M_2 (Holzapfel et al., 1966).[25]

4. Permissible Level of Aflatoxin in Animal Feed

With the improvement of the Codex Alimentarius, the European Commission (EC) recently introduced the recognized highest concentration/accumulation of aflatoxin in animal care 0.02 mg/kg, which is a complete feed for sheep, cows, goats, pigs, and poultry, 0.005 mg/kg, complete feed for calves and lambs 0.01 mg/kg (Directive 2002/98/EC of the European Parliament and of the Council of 27 January 2003, 2009). The permissible levels of aflatoxins in poultry feed are 20ng/g according to Food and Drug Administration & World Health Organization (Nakavuma et al., 2020).[33] FDA (Food and Drug Administration) of united state set the level of action for aflatoxin for food and feed. 20 ng g^{-1} or less for all human, animals and other species, >20-100 ng g^{-1} for breeding animal, >100-200 ng g^{-1} for pigs, >200-300 ng g^{-1} for beef cattle, >300-500 ng g^{-1} for ethanol production (non-animal uses) and >500 ng g^{-1} for non-salvageable are the range limits of corn products set by USFDA (United State Food and Drug Administration).

5. Factors Responsible for Aflatoxin Contamination in Animal Feed

The aflatoxin production is related directly to the growth of aflatoxigenic fungi. Many food sources in feed, environmental factors and enzymes are responsible for the contamination of aflatoxin in feed. The factors such as physical, biological, chemical, or nutritional that is responsible for the growth of the fungus are also suitable for aflatoxin production. Some of these are explained below:

5.1. Physical Factors

These include pH, temperature, humidity, moisture, and atmospheric gases. The optimum pH range for fungus producing aflatoxin is about 3-7 (Yoshinari et al., 2010).[51] Low pH causes minimization of growth of fungi but higher pH (6>pH>3) induces production of fungi and aflatoxin both (Eshelli et al., 2015).[19] The presence of light also; affects the production of aflatoxin and fungi. The production of aflatoxin increases in darkness whereas decreases in sunlight. (Rushing and Selim., 2019).[38] Higher content of moisture induces more contamination of aflatoxin. The optimal humidity, 85% causes the growth of aflatoxin but an increase of relative humidity to 95% causes its production level to a greater extent (Ding et al., 2015).[16] The optimum temperature range for growth of Aspergillus flavus is 28-37°C but it can tolerate temperatures 12-48°C also (Hawkins et al., 2005).[24]

The optimum temperature for aflatoxin production is 25-35°C (Siciliano et al., 2017).[41] A higher level of CO_2 and decreased level of O_2 causes inhibition of both fungus and aflatoxin Aflatoxin contamination is also due to region climate, type of soil, regulation both minimum and maximum, planted crop genotype, and the net evaporation on daily basis. Between 40^0N and 40^0S of the equator, the risk of contamination of aflatoxin is greater.

5.2. Biological Factors

Weeds, insects, and fungal species are major biological factors in this term. Aflatoxin production increases when the plant is stress-induced because of the growth of weed as a competitor. A type of fungal strain also affects its production. Aflatoxin production is fewer by Aspergillus flavus while higher by *Aspergillus parasiticus* (Manjunath and Mohana., 2018).[32] Aspergillus flavus has a saprobic character and is found enormously in soil; due to this it considers the main species for the production of aflatoxin. It can grow easily and readily

on dead plants and animals, field crops, killed insects, cotton, stored grains, and fodder (Kakde., 2012).[27] Contamination of monocot and dicot seeds is due to Aspergillus flavus.

5.3. Nutritional Factors

Several factors related to nutrition such as amino acids, lipids, carbohydrates, trace elements, and nitrogen play a role in the production of aflatoxin. Carbon is essential for the growth of fungus, so substrate enriched with carbohydrates such as ribose, glucose, sucrose, xylose, and glycerol aggravates the contamination of aflatoxin other than oil when compared. The production level of aflatoxin increases by nitrite and nitrates (Wang et al., 2017).[48] Lipophilic epoxy fatty acids lead to the biosynthesis of aflatoxin in toxic fungi and ergosterol oxidation causes the production of aflatoxin (Reverberi., 2014).[37] Aflatoxin production is promoted by the combination of glycine, glutamate, and alanine with metals i.e. bivalents such as zinc and magnesium (Bolu et al., 2014).[7] With the concentration of zinc of 20, 50, and 100 mg/L, the production of aflatoxin increases to 4, 5, and 19 times respectively (Liu et al., 2016).[29] Amino acids tyrosine induces the production of aflatoxin while tryptophan inhibits it (Chang et al., 2015).[10]

6. Determination of Aflatoxins in Animal Feed by Various Methods

Since 1970, there have been numerous chemical and biological ways for the detection and quantification of aflatoxins. The mycotoxins analysis has been standardized by different organizations like FDA, EPA, and AOAC. Using systematic methods, mycotoxins of even low quantity can also be determined in feed.

There is no homogeneity of mycotoxins in food and feed samples. Therefore, the process of extraction and preparation of the sample is critical to obtain the best analytical results. It consists of three steps:

1. Extraction
2. Purification or separation
3. Evaluation of detection

Different chromatographic and centrifugation methods like TLC, HPLC, GC, mass spectrometer, LC with a mass spectrometer, and ELISA are used for the purification of mycotoxins (Onji et al., 1998).[34]

7. Isolation and Determination of Aspergillus Flavus from Animal Feed Samples

About 0.2-0.5g of sample was taken that was crushed in sterile mortar and pestle and put on to the PDA (potato dextrose agar) medium and go for incubation at 28±20°C for three days (Almoammar et al., 2014).[4] The stock culture of the isolates of Aspergillus flavus was prepared by moving them on PDA plates and from them for more study, picked up single colonies, and transfer to the culture plate. The percentage of relative density and frequency of occurrence of fungi species were calculated by formula as below (Saleemi et al., 2010).[39]

RD (%) = no. of isolates of genus or species/ total no. of fungi isolated x 100

Fr (%) = no. of samples with genus or species/ total no. of samples x 100

8. Isolates of Fungi Screened for Production of Aflatoxin

The isolates taken from animal genes undergo screening on desiccated coconut agar for production of aflatoxin (Yazdani et al., 2010).[50] Incubation for 7 days at room temperature, isolates that absorbed and released very dark, moderate, and weak UV light (at 365nm) was capable of aflatoxin production.

9. Extraction

The most critical step in the process of extraction involves the extraction of interesting analytes without any changes. In a process of extraction, 10mL of 8:10 of methanol: water is added to 5g of finely crushed sample. It was put on a mechanical shaker for 30 minutes at an rpm of 400. In animal feed, aflatoxin B_1, B_2, G_1, G_2 is determined by ultra-high-performance liquid chromatography-tandem mass spectrometry applied by Grio et al. sonication extraction and by the use of 80:20 v/v of CAN/ water mixture. In this process, results for analytes of interests with low RSD values i.e. less than 20 % were in the range from 84-113% (Lopez Grio et al., 2010).[31]

10. Separation and Detection of Aflatoxin in Feed Samples

10.1. By Liquid Ammonia, Vapor Test

After incubation for 7 days at 28°C on PDA plates, turned the Petri dishes and on the cover of turned culture plate, added 2mL of concentrated ammonia solution and waited for 10-15 minutes for the release of ammonia vapor (Aikhersan et al., 2016).[2] The colour developed after the exposure of culture to ammonia vapors. Production of aflatoxin was positive on observing the colour change from yellow to dark yellow, pink, or reddish-brown. So such colour changes showed production of aflatoxin was negative.

10.2. Detection of Aflatoxin for Analysis

They are many innovative technologies are being used to detect the presence of aflatoxin. Some of the technologies are high-performance liquid chromatography (HPLC), Thin-layer chromatography (TLC), Enzymes linked Immunosorbent assay (ELISA), Fluorometry, quantitative and qualitative lateral flow assays, and LC/MS/MS so on (Rahmani et al., 2010).[36]

10.3. By Thin Layer Chromatography

The sample was crushed and weighed 50g grounded sample. Put in 500mL of the glass-stopper flask. Add 10mL and 200mL of citric acid solution and dichloromethane respectively. Shake for half an hour.

The mixture was filtered through filter paper in a 300mL flask. This flask contains 10g of Na_2SO_4. Gently allow contents to settle, and then the solution was refiltered through Whatman filter paper into a 100mL beaker. Samples were spotted on TLC plates. The TLC plates have coated gel of silica on a glass plate and are activated at 80°C for 1 hour in a hot air oven before use for analysis. This plate was put in a mobile phase tank that contains chloroform and acetone in a ratio of 88:12 (v/v) for half an hour.

The presence of aflatoxin was confirmed in the samples of contaminated animal feed by the presence of blue fluorescence under UV- light and compared with standard AFB1 sample (Sobolev and Dorner, 2002).[43] The contaminated feed samples have fewer RF values when compared to standard AFB1 RF values. TLC is very efficient for the detection of aflatoxin B_1 also.

10.4. High-Performance Liquid Chromatography

Due to the good power of separation, sensitivity, easy approach, and ability to use with various detection, HPLC has been used in the literature on a wide range. Reverse-phase chromatography is dominant over the normal phase for use in the detection of aflatoxins. Separation of B_1, B_2, G_1, G_2 aflatoxin in reverse phase depends on C-18 columns and 60:25:15 v/v of water: acetonitrile: methanol for mobile phase (Tarin et al., 2004).[46]

Fluorescent detectors are used for the detection of separated species. Due to the strong conjugation of oxygenated structures, aflatoxin B_2 and G_2 are highly fluorescent molecules. On the other hand, B_1 and G_1 are weak. Various fluorescent agents include TFA (trifluoroacetic acid), use of bromine or iodine, photochemical reactors, tandem mass detectors (MS/MS), electrochemical detectors, etc.

10.5. ELISA (Enzyme-Linked Immunosorbent Assay)

ELISA is a very specific and sensitive immunoassay used in this process. It requires small preparations of sample and permit sample analysis at high rates by using known concentration standard of aflatoxin. The working strategy of ELISA is that when a plate or column having arranged antibodies is exposed to aflatoxins, antibodies recognize antigens of aflatoxins and from the complex. This complex produces electrical, light, or any other measuring signal after interacting with a chromogenic substrate.

Quantification of poultry feed by ELISA is based on visual comparatively of intensity OD values of sample colour with the standard ones. Samples showed low colour amount than standard had more concentration of aflatoxin as compared to standard. On the other side, a sample having a large colour amount had less concentration of aflatoxin. Many countries have been set maximum permissible levels of aflatoxin in food to prevent its bad effects on human and animal health. FDA set the limit level of aflatoxin for feedstuff is 20µ/kg and for aflatoxin M_1, it is 0.5µ/kg. In the European Union, the limit level is 2microg/kg for aflatoxin B_1 in foodstuffs and 0.05microg/kg for aflatoxin M_1. The drawback of ELISA is the interaction of a similar chemical group of aflatoxin species with antibodies and causes interference in results (Zheng et al., 2005).[53] For the best and safe long-term use of ELISA kits, HRP (Horse Radish Peroxidase) is the most commonly used enzyme.

10.6. Detoxification of Aflatoxin in Animal Feed

Aflatoxin can be discovered worldwide in an assortment of food and feed wares particularly grains; the tainting with aflatoxin-creating growths and creation of the harmful in the items can happen in the field during capacity, transportation at practically all phases of the creation chain. In a completed creature feed, the defilement of fixing could cause the tainting of a whole feed cluster. It additionally leads to the deterioration of other feed shipments and fills in as the source of an organism in the feed business climate hard to kill co-event of various mycotoxins in completed feed could have significant adverse consequences on creature wellbeing (Alvarado et al., 2017).[5]

10.7. Aflatoxin Management in Pre and Post-harvest Animals Feed

Pre-harvest management of aflatoxin in animal feed based on good agricultural practices by the producers, regulation enforcement, and constant monitoring of aflatoxin in feed and feed ingredients. To resist the aflatoxin in animal feed molecular techniques are available and used to resist the attack and effect on animals feed and foodstuff. Some preharvest techniques are used to prevent aflatoxins great yield pivot with an appropriate length of soil rest, estate and reap of harvest during the right seasons and climates, and decrease of plant pressure (Aldred et al, 2004).[1]

11. Physical Methods

The virtual control of mycotoxins defile can be reduced by several physical methods such as colour sorting wise, mechanical separation, removal of the fines, and separation of fines during density segregation (Stepanik et al., 2007).[44] There are numerous methods of eliminating aflatoxin actually like tidy-up strategies, mechanical separation, and arranging therapy with heat, illumination, and treatment with ultrasonic instruments. Most sorts of aflatoxin are destroyed by high-temperature cooking and simmering (Levi et al., 1974).[28] The unfavourable mycotoxins are obliterated by gamma light and bright irradiation.

12. Chemical Method

Many chemical methods are useful against aflatoxins such as oxidizing agents, acids, reducing agents, bases salts, chlorinating substances, and other useful substances (Pascale and Visconti, 2008).[57] Ammoniation is a very useful method which is used for several years and it helps to decrease the number of aflatoxins in the feed. Many countries use the method of ammoniation to destroy aflatoxins. In some amounts of ammunition affect food quality. In food and feed mycotoxins levels are also reduced by the treatment with alkalization, acidification, and thermal (Bretz et al., 2006).[8] So many anti aflatoxins organisms are present such as aerobic and anaerobic bacteria's, fungal species such as yeast, protozoan's which is very useful to destroy the aflatoxin (Galtier and Alvinerie., 1976).[21]

13. Biological Method

Probiotics microorganism (Saccharomyces cerevisiae and Lactobacillus delbrueckii) was explored to diminish mycotoxins as organic control specialists and discovered the utilization of probiotics as an elective treatment to forestall aflatoxin creation in food elements (Silva et al., 2015).[42] Bacillus subtilis AF-1 of rhizobacterial nature is being used against the mycotoxins such as black molds.

14. Conclusions

Aflatoxins are carcinogenic compounds which may contaminate feed especially aflatoxin B_1 is potent carcinogen. When aflatoxins contaminated feed is taken by lactating animals like cows and goat etc., transmit toxin called aflatoxin M1: a metabolite of aflatoxin B_1. Aflatoxins contamination may be avoided by adapting quality assurance of feed samples and through quality control. There are physical, chemical and biological methods used for the decontamination of feed. Presence of aflatoxins in feed may affect animal as well as human health badly. The strict quality check on feed is direly needed to avoid hazardous health effects.

Conflicts of Interest

The authors declare no conflict of interest.

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