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Genotoxicity and metabolic pathway of aflatoxin isolate in contaminated poultry feed

Abstract

Aflatoxin is a nephrotoxic mycotoxin found in stale food which when consumed has some carcinogenic potentials. Aflatoxin presence has been identified in many foodstuffs all over the globe and with significantly higher frequency and concentrations coming from poultry feeds especially the locally formulated ones as a result of high cost of poultry feedstuff. Though foodstuffs are often contaminated with more than one mycotoxin, the focus of most research had centred on aflatoxin due to its wide occurrence and toxicology effect on the agro-morphometric parameters of poultry. The present studies reveals the major genes affected by aflatoxin ingestion in contaminated feed to include Tumour protein gene (TP53), Albumin protein gene (Alb), Spermatogenesis associated protein 5 (SPATA5), cytochrome p450 subfamily 3 polypeptide 4 and 7 protein genes (CYP3A4 and CYP3A7), glutathione s-transferase mu-1 protein gene (GSMU1) and minor genes such as the epoxide hydrolase 1 gene (EPHX 1), LACE 1 gene, XRCC1gene and cyclin 2 gene (CCGN2) whose expressions and functionality in the poultry has been silenced or activated leading to genotoxicity, highlights the biosynthesis and metabolic pathway of aflatoxins in poultry system and the toxin–protein interactions of aflatoxin ingested through contaminated poultry feed stuff as well as its co-occurrence with other mycotoxins in organisms as well on their combined toxicity. The metabolic pathway of aflatoxins B1, B2, G1 and G2 ingestion of aflatoxin in contaminated poultry feeds begins with the production of methyl-sterigmatocystin enzyme which pave the way for the complex, Sterigmatocystin which upon dimethylation produces versicolonin A (an enzyme which disrupts the colon) and versicolonin. The two enzymes reacts to form versiconial hemiactal acetate and producing 1‘5 Hydroxyaverantin both of which distorts the expression and functionality of Mycotoxin mixtures involving aflatoxin produced additive and synergistic effects in poultry birds suggesting that aflatoxins represent a significant health hazard. Hence, special attention should be given to poultry feedstuff screening and purification to prevent the associated toxicity that includes genotoxicity and the production of cancer-promoting mycotoxins.

Key words: tumour protein gene, albumin protein gene, CYP3A4 & CYP3A7, Aspergillusflavus, Aspergilusparasiticus

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Abbreviations: TP53, Tumour protein gene; Alb, Albumin protein gene; SPATA5, Spermatogenesis associated protein 5; CYP3A4 and CYP3A7, cytochrome p450 subfamily 3 polypeptide 4 and 7 protein genes; GSMU1, glutathione s-transferase mu-1 protein gene; EPHX 1, epoxide hydrolase 1 gene

Introduction

The livestock industry is an important subsector of the agricultural sector of Nigeria’s economy. The role of this sector cannot be over-emphasized considering the importance of animal protein in the diet of people and contribution from this sector to the gross domestic product. Livestock types found in Nigeria include cattle, sheep, goats, pigs, poultry, horses, donkeys and camel.[1] These animals are spread throughout various ecological zones in the country determined by environmental factors such as temperature, rainfall, humidity, light intensity etc. these factors determine whether an animal can or cannot survive within a specific environment. Livestock rearing is often considered a secondary occupation of many farmers in developing countries. Nevertheless, the importance of livestock in the livelihoods of the rural people cannot be underestimated. Households live on subsistent farming, often integrating crop production with livestock rearing, yielding multipurpose products and uses.[2] The increasing cost of keeping livestock have entrenched the art and science of local feed formulation and the use of low quality or substandard prepared feeds which attract contamination by microorganisms notably among which are mycotoxins expressed in forms of aflatoxins, ochratoxin, fumonisin, patulin and many others bacterial. Mycotoxins are toxic metabolites produced by certain fungi. They are always hazardous to man and domestic animals and had come to public interest since the past 30years. Mycotoxins are secondary toxic metabolites growing on food products, such as corn, peanuts, and wheat, among others. Exposure occurs predominantly by the ingestion of contaminated feeds.

When contaminated cereal such as corn, wheat, peanuts and sorghum, as well as other raw materials, are used in the preparation of animal feed. Among them, aflatoxin is a class of potent Mycotoxins produced mainly by Aspergillusflavus, Aspergilusparasiticus and occasionally by other Aspergilus species.[3] Aflatoxin constitutes a great threat to the health of animals and humans due to their teratogenic, carcinogenic, mutagenic and immunosuppressive effects.[4,5] Additionally, in terms of the livestock industry, aflatoxin causes a huge economic loss by retarding animal growth, increasing feed consumption and reduced meat production.[6,7] Among the various types of aflatoxins, aflatoxin B1 (AFB1) is known to be the most biologically active component. Aflatoxin, in the late 1950s and 1960s, were identified to be the cause of the turkey X disease in Great Britain.[8–10] Aflatoxin was identified as carcinogenic in rainbow trout.[11,12] In the United States, studies have implicated aflatoxin as the cause of epizootic hepatitis in dogs and as the cause of mouldy corn poisoning in pigs.[13] The immune system of the poultry is the first target to be influenced by the Mycotoxins. Immunosupression can be observed in poultry ingesting aflatoxins at levels below those that cause over symptomatology, and explained in parts, by atrophy of the bursa of fabricius, thymus, and spleen.[14]

Aflatoxin are resistant to food processing and therefore, could be found both in animal feed and foodstuff for direct consumption or processed foods, posing a risk for human health. All bird species are sensitive to aflatoxin toxicity and although they do not receive relatively high concentration of the feed and end lethally, low levels could also be deleterious after continuous intake. Growing birds, especially ducklings and turkey poultry are extremely sensitive to the toxic effect of aflatoxin. The total aflatoxin content in feed should not exceed 20ug/kg feed. Nevertheless, levels lower than 20ug/kg feed also induce lower resistance to diseases and stress.[15] The toxic effects of aflatoxin in domestic fowls are studied in detail with regards to their carcinogenic, teratogenic, mutagenic and growth inhibiting effects. The occurring haematological, biochemical (decreased serum, total protein, albumin, inorganic phosphorous, uric acid and total cholesterol and the values of heamatocrit, red blood cell counts, mean copuscular volume, haemoglobin, thrombocyte counts, percentage of monocyte counts, increased values of white blood cell and heterophils counts) Oguz, immunological depression in anti-bruscellaabortus antibodies) qureshi and morphological (hydropic generation in liver, significantly reduced in size and bursa of epithelium, intraepithelial cysts oedema in the intraepithelial areas and heterophil infiltrations: slightly lymphoid depletion of periateriolar lymphoid tissues in spleen; pale kidney with degeneration and/or necrosis of tubular epithelium. There are also grave effects of aflatoxins on the major and minor protein genes in poultry which further creates room for genetic disorders.

The morphological changes in the spleen are characterized with lymphatic degeneration, fatty dystrophy and haemorrhagic foci, cognitive event in the red pulp, reduction of lymphocytes, vascular dystrophy, and depletion of lymphoid cells, reticular cell hyperplasia, lymph cytolysis and increased germinal cell counts. A number of strategies for detoxification of Mycotoxins contaminated feeds are proposed and have included physical separation, heat inactivation, irradiation, microbial degradation, treatment with chemicals. Regardless of that, some of them reduce also the bioavailability of amino acids and/or minerals in the poultry birds. The importance of aflatoxin in poultry production cannot be overemphasized and thus created the heightened need for this research.

Materials and methods

a. Isolation of mycotoxin

Potato dextrose agar plate was prepared and collected. Moulded poultry feed (growers mash) was serially diluted in saline solution. The samples were then inoculated into the agar plates. The plates (plate 1) were further inoculated at $28^{\pm}2^{0}$C for 3-5days. After incubation, the total numbers of fungal population per gram of the feed were estimated and fungal species identified.

b. Aflatoxin isolation

The Mycotoxin from *Aspergillus flavus* called Aflatoxin was isolated from collected moulded poultry growers mash. Other materials used for the isolation process included potato dextrose agar, saline solution, chloroform, sodium bicarbonate solution, ethyl acetate, formic acid, toluene, 1% p-dimethyl aminobenaldehyde, petri dishes, incubator, microscope, pH scale, conical flask, Whatmann No. 1 filter paper, water bath, evaporator and silica gel.

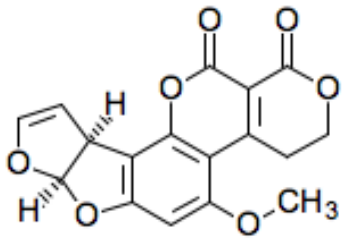
The concentrated culture filtrate was shaken repeatedly with 100ml volume of chloroform and the extraction repeated 2 or 3 times. The chloroform extracts was combined and filtered through Whatmann No.1 filter paper. From the filtered chloroform extract, the toxins were extracted with sodium bicarbonate solution by shaking the chloroform extract several times with 0.5M sodium bicarbonate solution. All lipid materials were separated by filtration after keeping the sodium bicarbonate overnight in a separating funnel. Finally, the pH of the solution was brought down to 2.0 and the toxins were extracted from the concentrates into chloroform by repeated extraction with aliquots of chloroform. The extracts were cooled and concentrated and the crude toxins isolated.

c. Determination of genotoxicity, biosynthesis and metabolic pathway of aflatoxin in contaminated feedstuff

Metabolic pathway and genotoxicity prediction was performed using Toxtree 2.6. and the Chembi KEGG online programme resources. The associated effect of aflatoxins on the target major and minor genes in terms of absorption, metabolism, distribution, excretion and toxicity. The expasy.org, click2drug, pathway databases stitch online resource programme was used to determine the aflatoxin genome (genotoxicty) interactions for the associated genetic, molecular, biological and cellular processes.

d. Chemical structure of aflatoxin and compound identifier

Aflatoxins are a group of closely related toxic metabolites that are designated mycotoxins. They are produced by Aspergillus flavus and Aspergillus parasiticus. Members of the group include aflatoxin B1, aflatoxin b2, aflatoxin G1, aflatoxin G2, aflatoxin M1 and aflatoxin M2 (MeSH) (328.3g/mol).



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Results

i. Aflatoxin chemical and Protein Interactions in Poultry and humans.

ii. Genetic impairment of Protein functions and expressions by aflatoxins in contaminated poultry feeds.

i. CYP3A4 belongs to the cytochrome P450, family 3, subfamily A, polypeptide 4 protein gene (Figure 3). The cytochrome P450 are a group of the heme-thiolate monooxygenases found in the liver microsomes. This enzyme is involved in an NADPH –dependent electron transport pathway. It performs a variety

of oxidation reactions (e.g controls caffeine 8-oxidation, omeprazole sulphoxidation, midazolam 1-hydroxylation and midazolam 4 hydroxylation of structurally unrelated compounds including steroids, fatty acids and xenobiotics. It acts as a 1,8-cineole 2-exomonooxygenase. The protein gene also hydrolates etoposide and contains 503 amino acids. The active exon site for this protein is 20-349KDa in the genome. Aflatoxin disrupts the oxidations of toxic harmful substances which enters the liver such as steroid, unsaturated fatty acids and xenobiotics. Aflatoxin also inhibits the NADPH –dependent electron transport pathway thus disrupting biochemical processes which requires high energy for complete cellular activities.

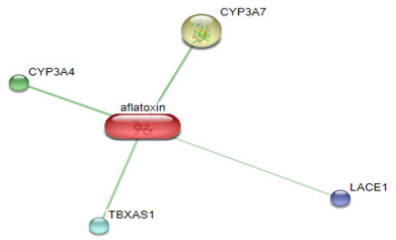

Figure 1 Aflatoxin chemical – protein interactions in poultry birds.

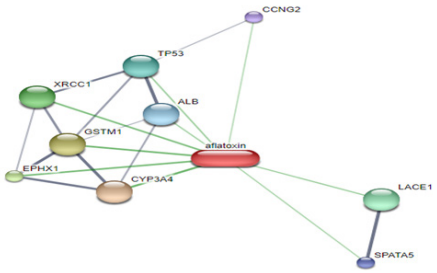

Figure 2 Aflatoxin chemical – protein interactions in humans**.**

Figure 3 3D protein structure of the cyp3a4 cytochrome p53 family with 503 amino acids.

ii. CYP3A7 belongs to the cytochrome P450, family 3, subfamily A, polypeptide 7 chain (Figure 4) which is similar in action but at a lower dimension to the polypeptide 4 chain. These cytochrome P450 are also a group of the heme-thiolate monooxygenases found in the liver microsomes. This enzyme is also involved in an NADPH –dependent electron transport pathway. The active exon site for this protein is 31-505KDa in the genome. Aflatoxin disrupts the oxidations of toxic harmful substances which enters the liver such as steroid and fatty acids. Aflatoxin also inhibits the NADPH –dependent electron transport for these pathway thus disenabling and uncoupling biochemical processes which requires high energy for complete coupling and complex formation.

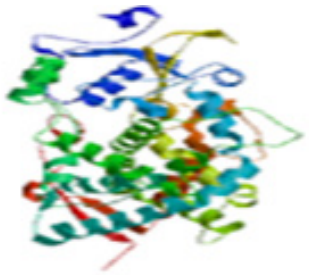

Figure 4 3D protein structure of the CYP3A7 cytochrome P53 family with 505 amino acids.

iii. GSTM1 is glutathione S-transferase mu-1 (Figure 5). This protein form a conjugant with aflatoxin, thus reducing glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles or substances which is useless to the animal system.glutathione is very useful in the animal system as an immune booster and a correctional protein to many unstable proteins in the body. It active exon site is found within the first 1 and last 218 KDa which corresponds to the exact size of the gene (218aa). Aflatoxin reduces the metabolism and functionality of glutathione in the body thus weakening the immune system and increasing the susceptibility of the organisms to other infections.

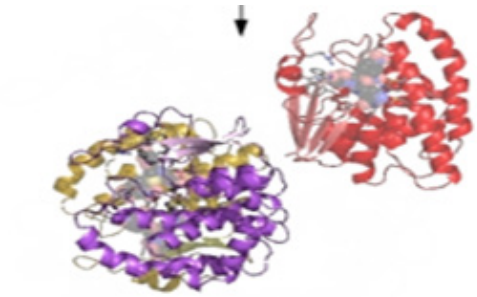

Figure 5 3D protein structure of the GMST1 glutathione S –transferase mu -1 gene.

iv. XRCC1 is an X-ray repair complementing defective repair protein gene (Figure 6) found in the Chinese hamster cells 1. It functions in the corrections of defective DNA strand-break repair and sister chromatid exchange following treatment with ionizing radiation and alkylating agents. The exon active site of this protein gene is found between 1 and 183KDa out of the 633aa protein size. The presence of aflatoxins in body impairs the repair functions of this protein gene. Alkylating agents are de-alkylated by aflatoxins and the defective repair protein is denatured in the presence of aflatoxin compound.

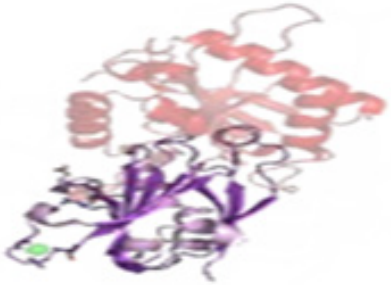

Figure 6 3D protein structure of the XRCC1 X-ray repair complementing detective protein gene.

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v. EPHX1 is epoxide hydrolase 1 (Figure 7**)**, which reacts with microsomal xenobiotics (human toxins) ingested alongside aflatoxins in feedstuff, thus reducing their negative effects. Contact with aflatoxin mycotoxin will impede the role of the EPHX1 protein and invariably reduce the expression and functionality of the protein gene.

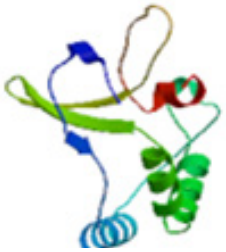

Figure 7 3D protein structure of the EPHX1; epoxide hydrolase 1 gene.

vi. LACE 1 is lactation elevation 1 protein gene (Figure 8**)** and is responsible for the development of female reproductive system in poultry birds. Contact with aflatoxin mycotoxin will impede the role of the LACE 1 protein and invariably reduce the expression and functionality of the protein gene in poultry birds.

Figure 8 3D protein structure of the LACE 1; lactation elevation 1 protein gene.

vii. TP53 is the tumour protein 53 (Figure 9**)** which acts as a tumour suppressor in many tumour types. It induces growth arrest or apoptosis depending on the physiological circumstances and cell types. It is involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. Apoptosis induction seems to be mediated either by stimulation of BAX and FAS antigen expression or by repression of Bcl-2. Contact with aflatoxin mycotoxin will impede the role of the TP53 protein and invariably reduce the expression and functionality of the protein gene and prompting cancer-enhancing oncogenes.

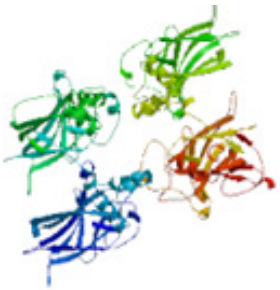

Figure 9 3D protein structure of the TP53 protein gene; tumour protein.

viii. ALB is Albumin protein gene (Figure 10**)** which is very critical in the formation of egg white protein in poultry birds. Contact with aflatoxin mycotoxin will distort the development of egg white albumin protein and invariably reduce the size, development, expression and functionality of the protein gene.

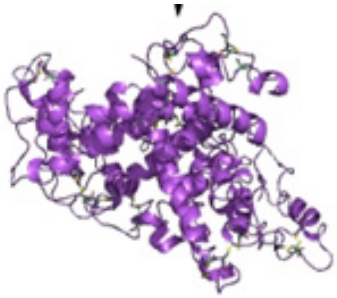

Figure 10 3D protein structure of the Albumin protein gene.

ix. SPATA5 is spermatogenesis associated protein 5 (Figure 11**)** which is involved in morphological and functional mitochondrial transformations during spermatogenesis in the poultry birds. SPATA5 contamination with aflatoxin mycotoxin will impede the role of the spermatozoa production by the cocks and invariably reduce the expression and functionality of the protein gene.

Figure 11 3D protein structure of the SPATA5 protein gene; spermatogenesis associated protein gene.

x. CCNG2 is cyclin G2 protein (Figure 12**)** which plays a crucial role in growth, regulation and in negative regulation of cell cycle procession. Contact with aflatoxin mycotoxin will impede the role of the cyclin G2 protein and invariably reduce the expression and functionality of the protein gene.

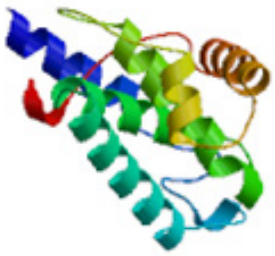

Figure 12 3D protein structure of the CCNG2 protein gene; cyclin G2 protein gene.

xi. Coexpression of TP53 and CCNG2 in Gallus gallus and Danio renio as influenced by aflatoxins

The predicted association between TP53 and CCNG2 was carried out based on the observed coexpression of the similarity or homologue observed in Danio regio. The TP53 –Tumor protein p53 acts as a tumor suppressor in many tumor types. It induces growth arrest or causes apoptosis (cell death) depending on the physiological circumstances and the cell type. It also involves the cell cycle regulation as a tran-activator that acts to negatively regulate cell division by controlling a

set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. Apoptosis induction seems to be mediated either by stimulation of BAX and FAS antigen expression or by repression of Bcl-2 expression showing a coexpression score of 0.766 (Table 1).

Table 1 Functional analysis of the gene sets

Functional analysis of the gene sets	Set of genes involved	False discovery rate
Genes that are involve in the metabolism of xenobiotics using Cytochrome P450.	3	0.000678
Genes associated with chemical carcinogenesis.	3	0.000678
Genes that plays crucial role in drug metabolism and Cytochrome P450 activities	2	0.0276
Genes that establishes the P53 signalling pathway	2	0.0276
Genes that plays critical role in bile secretion.	2	0.0276

xii. Metabolic pathway and biosynthesis of aflatoxin contaminated feed in poultry

The metabolic pathway of aflatoxins B1, B2, G1 and G2 are presented in figure 13 above. The ingestion of aflatoxin B1 in contaminated poultry feeds begins the production of methyl-sterigmatocystin enzyme which pave the way for the complex, Sterigmatocystin which upon demethylation produces versicolonin A (an enzyme which disrupts the colon) and versicolonin B. The two enzymes reacts to form versiconial hemiactal acetate and producing 1‘5 hydroxyaverantin.

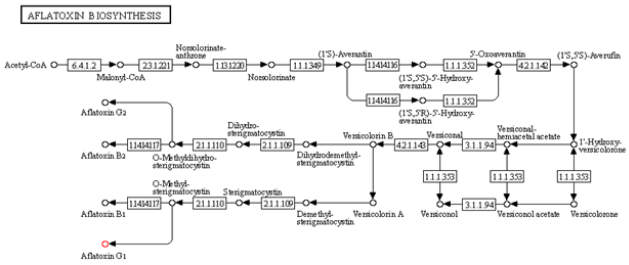

Figure 13 Metabolic pathway of aflatoxins in host animal circulatory and enzymatic reactions.

Control of mycotoxicosis depends on the safeguarding of mycelial growth in the feedstuff, and on removing toxin contaminated feed. Unfortunately, aflatoxin is most likely and always found in contaminated feeds and foodstuff. The food and agriculture organisation (FAO) had posited that not less than 25% of global cereals are contaminated with aflatoxins. Hence, researching for procedures for detoxification becomes imminent.[16] Though such processes should be able to detoxify aflatoxins to below regulatory limits and also prevent the production of additional compound from aflatoxin metabolism, and as well as maintaining the nutritional composition of the detoxified feedstuff. A good toxin binder should restore the nutritional values of aflatoxin contaminated feed. The quality of a good toxin binder is expressed in several means including, knowledge of their binding capacity, the absorption efficacy, the activation time and rate of Inclusion of aflatoxin contaminant in the feedstuff.[17,18]

Several methods have been developed in pursuant to their ability to inactivate aflatoxin in contaminated feedstuffs, with the sole aim of either removing or destroying the toxin and which may take the forms of physical, biological and chemical approaches. The approach adopted fully depends on the available know-how.

Discussion

The general decrease in PCV, RBC, WBC, and Hb of broilers fed diets that contained graded levels of AFB1 particularly as the level increases from 25% to 35% to 45% to 55% shows that the toxin may contain blood diminishing factors that may have stimulated the shortage of blood production by the broilers.[19] demonstrated decrease in Hb, RBC and PCV due to decreased mineral content of feed which is an indispensible factor of erythropoesis. The gradual drop in WBC as the inclusion levels increased suggest that AFB has negative effect to the immune system of the broilers. These haematological parameters fall within the low physiological ranges as established by[19,20]

The commercial poultry in Nigeria is largely private sector driven. The government only provides policy support. Household poultry flock size appears to be larger on the average than in the previous decade probably because of some elements of commercial poultry being introduced into it. In other to increased and maximize profit, most farmers and practitioners go into feed formulation with a resultant aflatoxin contamination. Organic acid is considered one of the most widely used food additives, which is commonly used as a preservative, acidulant, pH control agent, flavour enhancer and antioxidant in many foods. Organic acid is one of the important factors responsible for the characteristics smell and sour taste revealing its antibacterial and antifungal properties.[21] Organic acids have been used to decrease the growth of aflatoxins and reduce secretion of harmful fungi. In addition, they have many effect on poultry health, including growth performance, decrease feed conversion ratio, and in haematological and pathological indices through the detoxification of aflatoxin.[22]

The use of mycotoxin binders such as biological methods, the microorganisms and their metabolites to be tailored to eliminate aflatoxins. This can be a good promising strategy due to its specificity, efficiency, and environmentally friendly method of detoxification.[22] Some microbes including fungal and bacterial isolate, such as live yeast, Sacchromyces cerevisiae.[19,22] Flavo bacterium aurantiacum,[20] Stenotrophomonas Maltophilia,[21]Myxococus fulvus[16] and Aspergilus Niger[17,22] were reported to effectively biodegrade aflatoxin in vitro. However, little is known about their efficiency in the biodegradation of aflatoxin and effect of aflatoxicosis in vivo. As a biological product, several studies have revealed that esterified glucomannan derieved from cell wall of Sachromyces cerevisiae[20,22] have shown considerable promise in countering aflatoxins. Studies of using biological Mycotoxins based on S. Cerevisae, showed that using that binder in contaminated feeds was responsible for reducing liver residual aflatoxin levels,[21,22] and in ameliorating the negative effect of aflatoxin on Newcastle antibody production.[22,23]

Conclusion

The study concludes that the major genes affected (genotoxicity) by aflatoxin ingestion in contaminated feed are the Tumour protein gene (TP53), Albumin protein gene (Alb), Spermatogenesis associated protein 5 (SPATA5), cytochrome p450 subfamily 3 polypeptide 4 and 7 protein genes (CYP3A4 and CYP3A7), glutathione s-transferase mu-1 protein gene (GSMU1) and minor genes such as the epoxide hydrolase 1 gene (EPHX 1), LACE 1 gene, XRCC1gene and cyclin 2 gene (CCGN2) whose expressions and functionality in the poultry has been silenced or activated leading to genotoxicity.

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Highlights of the biosynthesis and metabolic pathway of aflatoxins B1, B2, G1 and G2 ingestion in contaminated poultry feeds induces the production of methyl-sterigmatocystin enzyme which pave the way for the complex, Sterigmatocystin which upon dimethylation produces versicolonin A (an enzyme which disrupts the colon) and versicolonin. The two enzymes reacts to form versiconial hemiactal acetate and producing 1'5 Hydroxyaverantin both of which are cancer promoting substances and also distorts the expression and functionality of SPATA5, CYP3A4 and GSMU1 genes. Hence, there is urgent need for special attention to be given to poultry feedstuff screening and purification to prevent the associated toxicity that includes genotoxicity and the production of cancer-promoting mycotoxins. Considering the numerous practitioners of rural poultry in Nigeria, a special and urgent attention is needed for an effective control of Aflatoxin contamination of poultry feed to minimize the implications for the economy and public health.

Recommendations

Re-evaluate the options for the control of Aflatoxin with a view to determining which of the two options of either eradication or vaccination that will provide more comprehensive and long lasting solutions. Adequate poultry feed storage strategies should be developed and adopted to avoid the contamination of these feed by aflatoxin which could also affect human health as well as the health of the birds.

Acknowledgments

None.

Conflicts of interest

Authors declared no conflicts of interest.

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None.

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