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Aflatoxins: A Comprehensive Overview

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Abstract:

Aflatoxins continue to raise health concerns as unavoidable and widespread natural contaminants of foods and feeds with serious impact on health, agricultural and livestock productivity, and food safety. They are secondary metabolites produced by *Aspergillus* species distributed on three main sections of the genus (section *Flavi*, section *Ochraceorosei*, and section *Nidulantes*). Aflatoxin-producing species, mainly *A. flavus* and *A. parasiticus* thrive under hot and humid conditions in the field or during storage, which are met in tropical and sub-tropical regions. Poor economic status of a country exacerbates the risk and the extent of crop contamination due to faulty storage conditions that are usually suitable for mold growth and mycotoxin production; temperature of 22 to 29°C and water activity of 0.90 to 0.99. This situation paralleled the prevalence of high liver cancer and the occasional acute aflatoxicosis episodes that have been associated with these regions. Few of the presently known aflatoxins (>18) have been sufficiently studied for their incidence, health-risk, and mechanisms of toxicity to allow effective intervention and control means that would significantly and sustainably reduce their incidence and adverse effects on health and economy. Among these, aflatoxin B1 (AFB1) has by far been the most studied; and yet, many aspects of the range and mechanisms of the diseases it causes remain to be elucidated. Its mutagenicity, tumorigenicity, and carcinogenicity, which are the best known still suffer from many limitations regarding the relative contribution of the oxidative stress and the reactive epoxide derivative (Aflatoxin-exo 8,9-epoxide) in the induction of the diseases, as well as its metabolic and synthesis pathways. Additionally, despite the well-established additive effects for carcinogenicity between AFB1 and other risk factors, e.g., hepatitis viruses B and C, and the algal hepatotoxic microcystins, the mechanisms of this synergy remain unclear. A review of publications on the incidence and concentrations of aflatoxins in selected foods and feeds from countries whose crops are classically known for their highest contamination with aflatoxins, reveals that despite the intensive efforts made to reduce such an incidence, there has been no clear tendency, with the possible exception of South Africa, towards sustained improvements. The levels and incidence are essentially influenced by the rainfall and temperature during the cultivation year or two successive years with alternating dry and wet seasons. This review aimed to update the main aspects of aflatoxin production, occurrence and incidence in selected countries, and associated adverse health effects. In addition to AFB1 which was the main focus of the review, other aflatoxins were addressed whenever relevant data were available.

Key words: Aflatoxins, incidence; Sub-Saharan Africa; Southeast Asia; tumorigenicity; carcinogenicity; acute toxicity; immunogenicity; genotoxicity

1 Introduction

Mycotoxins are among the microbial toxins of most concern to public health, and they represent a barrier to a wider international trade of agri-food products and an important obstacle in the face of the harmonization of regulatory standards globally, as was discussed earlier [1]. They are produced by various mould species as low-molecular-weight non-immunogenic secondary metabolites whose occurrence has been reported in virtually all foods and feeds [2-3]. Currently, there are more than 450 different known types of mycotoxins and their metabolites, which have been associated with toxicological effects of varying severity degrees spanning from mild gastroenteritis to deadly cancer diseases [4-5]. Aflatoxins produced mainly by *Aspergillus* species are the most toxic mycotoxins eliciting acute and chronic toxicities, the most severe and notorious of which are genotoxicity, mutagenicity, and immunotoxicity. Their toxicological status as human carcinogens is now beyond doubt, and it has been recognized by the International Agency for Research on Cancer (IARC) [6].

Although aflatoxins are of a global concern, their negative impact on health, economy, and social life is greater in developing countries located in the tropical and sub-tropical regions. Agricultural products from Sub-Sahara African countries, e.g. The Gambia, Uganda, Kenya, and Tanzania, and Southeast Asian countries, e.g., China, Thailand, Vietnam, and Indonesia, have classically been associated with the highest incidence of aflatoxins, which paralleled the highest incidence of hepatocellular carcinoma and the occurrence of acute aflatoxicosis outbreak episodes in the region [7]. As matter of fact, these regions have been the primary destination for scientists to carry out epidemiological studies on the relationship between the dietary exposure to aflatoxins and liver cancer, which contributed greatly to the establishment of aflatoxins as an aetiological factor of the disease in humans. Four major types of aflatoxins [aflatoxin B1, aflatoxin B2 (AFB1), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2)] are the best known and the most studied among more than 18 different types and metabolites presently identified.

This work aims to present an up-to-date overview on the structural diversity, the toxicity, ecological parameters for the production, and occurrence in foods and feeds of as many as possible aflatoxins or metabolite whenever relevant data are available. However, special emphasis was put on aflatoxin B1 (AFB1) as the flagship aflatoxin for being the most toxic and widespread. A review of the recent publications on aflatoxin occurrence in foods and feeds in selected Sub-Sahara African and Southeast Asian countries known for their highest dietary exposure is also presented.

2 Production, Structural Diversity, and Main Toxicological Properties of Aflatoxins.

2.1 Aflatoxin-producing Molds: Taxonomical Elements and Atoxigenic Strains

The production of aflatoxins has been reported in members of three sections of *Aspergillus* genus; section *Flavi* (B- and G-type aflatoxins), section *Ochraceorosei* (aflatoxins B1 and B2), and section *Nidulantes* (formerly *Emericella* genus; aflatoxin B1) [8]. However, species of section *Flavi* are the most common and potent aflatoxin-producing moulds, with *A. flavus* and *A. parasiticus* being the most frequently encountered in agricultural products because of their widespread distribution in the agricultural environment and their versatility to grow and produce aflatoxins under different ecological conditions [9-11]. A recent classification based on a polyphasic approach revealed that 18 species out of 33 of the section *Flavi* are aflatoxigenic and that each of 16 species is able to produce the 4 major aflatoxins (AFB1, AFB2, AFG1, and AFG2), while the other 2 species produce either AFB1 alone (*A. togoensis*) or both AFB1 and AFB2 (*A. pseudotamarii*) [11] (Table 1). The latter authors noted that *A. flavus* strains of Korean origin produce G aflatoxins, contrary to the prevailing view that this species strictly produces B aflatoxins [12-13]. In fact, the production of the G aflatoxins by *A. flavus* was reported when these aflatoxins were first discovered [14], but a controversy was raised when G-aflatoxin-producing strains NRRL 2999, 3000, and 3145, originally identified as *A. flavus*, were re-classified as *A. parasiticus* [8,15]. Subsequently, Wicklow and Shotwell [16] confirmed the production of B and G aflatoxins by other strains of *A. flavus*; NRRL strains 3357, 6412, 6554,

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6555, and 13003. Yet, the inability of *A. flavus* to produce the G aflatoxins was later reiterated and evidenced by genetic analysis relating indel (short insertions or deletions) mutations in the *cypA*/*norB* region in *A. flavus* to the impairment of the expression of genes coding for P450 monooxygenase enzyme required for the biosynthesis of G aflatoxins [17-19]. However, it was suggested that this mutation does not occur in all strains, and some *A. flavus* strains can produce B or G aflatoxins depending on the morphotype (S or L) and on the phylogenetic group (I or II) to which they belong. The morphotypes are defined by the size of sclerotia formed by the strains; "S" for small sclerotia (<400 µ in diameter) and "L" for large sclerotia (diameter >400 µ). In this regard, it was admitted that the phylogenetic group I includes both S- and L-morphotype strains which produce only the B aflatoxins, while group II contains only the S-morphotype strains which produce both B and G aflatoxins [20]. However, it was later demonstrated that the phylogenetic group I strains produce both B and G aflatoxins regardless of the morphotype, and that the phylogenetic group II is not restricted to the S-morphotype strains but contains also the "L" morphotype strains [10]. Furthermore, it was demonstrated that some S-trains (S_{BG}) produce both B and G aflatoxins, while others (S_B) produce only B aflatoxins [21]. Recent taxonomy studies using a combination of advanced analytical techniques confirmed that *A. flavus* can indeed produce B and G aflatoxins irrespective of the morphotype [10-11]. Notwithstanding, it is well established that S-morphotype strains are more aflatoxigenic than their L-morphotype counterparts, and they accumulate larger amounts of aflatoxins regardless of the aflatoxin type [10,21-22]. This was explained by the fact that the production of aflatoxins increases as the size of sclerotia decreases during their formation [22]. Indeed, in the low-elevation regions in Kenya where the S-morphotype is predominating (>90%), the concentration of aflatoxin B1 in maize was reported to exceed 1000 µg/kg [18]. This was practically illustrated by the higher incidence of deadly acute aflatoxicosis in these regions compared with those where the S-morphotype strains are less common [23]. Currently, there is an increased research interest in the identification and characterisation of atoxigenic strains of *A. flavus* belonging to vegetative compatibility groups (VCG) that can compete with aflatoxigenic strains and colonize fields where susceptible crops to aflatoxin contamination are cultivated. Such a trend emphasises the need to design appropriate and easy screening and characterization techniques to separate toxigenic from atoxigenic *Aspergillus* strains on the basis of vegetative compatibility analysis. This will help understand the fitness of atoxigenic vs toxigenic mold strains and their adaptation mechanisms to various environmental and soil conditions in order to adopt effective and environment-friendly biocontrol means, i.e., colonization of fields in various AEZ and soil types by selected atoxigenic strains of different VCGs to displace the naturally occurring toxigenic strains. The first application of this technology was done by the US Agricultural Research Service of the Department of Agriculture (USDA-ARS) in 2003 on cotton using atoxigenic *A. flavus* AF36 strain, which was then registered with the US Environmental Protection Agency (USEPA) [24]. The following year, the same organism patented this technology as a biocontrol product that was licenced by a relevant industry under the trade name of afla-guard® [25]. As this technology proved to be an efficient biocontrol means to mitigate aflatoxin contamination in various crops, studies have been conducted in different countries and regions of the world to screen for proficient strains and well adapted to specific soils and AEZs. In Ghana, atoxigenic African *A. flavus* VCG (AAV) strains isolated from three different agroecological zones (AEZ) reduced aflatoxin contamination of maize and peanut by 87–98% in laboratory assays, and successfully displaced toxigenic *A. flavus* strains in field trials where crops obtained from treated grains contained 50-100% less aflatoxin at harvest than their untreated counterparts [26]. In Northern Italy, co-inoculation of maize ears with an endemic atoxigenic strain *A. flavus* A2085 of the VCG IT019 group and an aflatoxigenic strain (A2092) of the same species was reported to reduce the concentration of AFB1 by 93-98% compared with ears inoculated with the aflatoxigenic strain alone [27]. In field trials, the atoxigenic strain A2085 reduced the concentration of AFB1 in crops at harvest from treated fields by an average of 92.3% compared with the crops from non-treated fields. This strain is now marketed as a biopesticide under the trade name of AF-X1™. Other successful field trials of different scales have been

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reported in different countries emphasising the anticipated success of this promising technology in the protection of crops against aflatoxin contamination for field to consumption [25-26,28-29].

2.2 *Physical, Chemical, and Toxicological properties of Aflatoxins*

More than 18 different aflatoxin types are presently known to occur naturally or as a result of carry over phenomenon in feeds and foods (Table 1). There are about 13 types of aflatoxins that are naturally produced by toxigenic moulds, some of which can be metabolised by human, animals, or other microorganisms to generate derivatives that retain toxicity, although usually with a lower potency compared with the parent molecules. AFB1, AFB2, AFG1, and AFG2 are of the most concern to economy and public health due to their high incidence and high toxicities, especially AFB1. Meanwhile, aflatoxin M1 (AFM1) is of special concern to the safety of dairy products because it is usually carried over in milk of lactating animals fed on feed contaminated with aflatoxin B1 in addition to its high toxicity and potential carcinogenicity in humans [2]. However, the other aflatoxins should not be overlooked because of their intrinsic toxicity, which may not be negligible, or because they can readily invert to the most potent AFB1. They can also be intermediates for the biosynthesis of more toxic mycotoxins [30-32]. Table 2 summarises physicochemical and toxicological properties of the main presently known aflatoxins.

159 **Table 1:** Origins of aflatoxins and the most exposed products to contamination.

Aflatoxin	Source	Frequently contaminated products	Reference
Difurocoumarocyclopentenone			
Aflatoxin B1	Section *Flavi*: *A. flavus, A. pseudotamarii, A. togoensis. A. aflatoxiformans, A. austwickii, A. cerealis, A. arachidicola, A. minisclerotigenes, A. mottae, A. luteovirescens (formerly A. bombycis), A. nomius, A. novoparasiticus, A. parasiticus, A. pipericola, A. pseudocaelatus, A. pseudonomius, and A. sergii, and A. transmontanensis* Section *Ochraceorosei*: *A. ochraceoroseus and A. rambellii* Section Nidulantes*: A. astellatus, A. miraensis, A. olivicola, and A. venezuelensis*	Cereals (e.g., sorghum, rice, corn, wheat, barely), oil seeds (e.g., cotton seed, oilseed rape, sunflower seed), nuts (e.g., peanuts, groundnut, pistachio), spices (e.g., turmeric, black and red pepper, ginger, allspices), meats, dairy products, fruit juices, dried fruits, eggs, and feeds and foods derived from these products	[8,11,33-35]
Aflatoxin B2	Section Flavi: *A. flavus, A. pseudotamarii, A. aflatoxiformans, A. austwickii, A. cerealis, A. arachidicola, A. minisclerotigenes, A. mottae, A. luteovirescens, A. nomius, A. novoparasiticus, A. parasiticus, A. pipericola, A. pseudocaelatus, A. pseudonomius, A. sergii, A. transmontanensis,* Section *Ochraceorosei: A. ochraceoroseus and A. rambellii*	Cereals (e.g., sorghum, rice, corn, wheat, barely), oil seeds (e.g., cotton seed, oilseed rape, sunflower seed), nuts (e.g., peanuts, groundnut, pistachio), spices (e.g., turmeric, black and red pepper, ginger), meats, dairy products, fruit juices, dried fruits, eggs, and feeds and foods derived from these products	[8,11,33-35]
Aflatoxin $B2_a$	Hydroxylated metabolite of aflatoxin B1 obtained by water addition to the double bond of the terminal furan under acidic conditions in the liver, the stomach or soil (no evidence for the involvement of specific enzymes) Naturally produced by *A. flavus*, and *A. parasiticus*	NA	[36-39]
Aflatoxin M1	Hydroxylated metabolite of aflatoxin B1 by hepatic microsomal mixed-function oxidase system (MFO), mainly cytochromes, in the liver of mammals Produced in vitro from aflatoxin B1 by liver homogenates Naturally produced by *A. flavus* and *A. parasiticus*	Milk (including human milk) and dairy products Meat products (kidney, liver) Mouldy groundnut and corn	[33,40-41]
Aflatoxin M2	Hydroxylated metabolite of B2 by hepatic microsomal MFO of mammals Naturally produced by *A. parasiticus*	Idem as aflatoxin M1	[33,41]

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Aflatoxin M2[a]	Hydration of the terminal furan ring of aflatoxin M1 in dilute acid to yield an hemiketal derivative *In vitro* in liver homogenates	Milk and dairy products	[42]
Aflatoxin P1	Demethylated metabolite of aflatoxin B1 by liver microsomal oxidase–catalysed O-demethylase	Mainly excreted in the urine (humans and animals). Dairy products	[33,40,43-44]
Aflatoxin Q1	Hydroxylated metabolite of aflatoxin B1 by microsomal enzymes in the liver of higher vertebrates and poultry (main aflatoxin B1 metabolite in monkey)	Assumed to be in edible parts of bovine fed on aflatoxin B1-contaminated feed	[33,40,45]
Aflatoxin Q2[a]	Acid hydration of aflatoxin Q1	NA	[46]
Aflatoxicol (R_0)	Metabolite of aflatoxin B1 formed by a reversible reduction of the pentanone group in humans, animals and numerous bacteria and molds In vitro biotransformation of aflatoxin B1 by a soluble cytoplasm reductase system in fish, rat and human liver preparations Naturally produced by *A. flavus* and *A. parasiticus*	Mainly avian products (major metabolite in avian species fed on B1-contaminated feed). Dairy products Does not accumulate in edible parts of bovine and swine fed on aflatoxin B1-contaminated feed	[43-44,47-53]
Aflatoxicol M1	Reduced metabolite of aflatoxin B1, aflatoxin R_0, or aflatoxin M1 catalysed by soluble NADPH-dependent reductases in the liver	Milk and dairy products	[33]
Aflatoxicol H1	Reduced metabolite of aflatoxin B1 and aflatoxin Q1 catalysed by soluble NADPH-dependent reductases in the liver	Milk and dairy products	[33,54]
Difurocoumarolactone			
Aflatoxin G1	*A. flavus*[a], *A. aflatoxiformans, A. austwickii, A. cerealis, A. arachidicola, A. minisclerotigenes, A. mottae, A. luteovirescens, A. nomius, A. novoparasiticus, A. parasiticus, A. pipericola, A. pseudocaelatus, A. pseudonomius, A. sergii, A. transmontanensis,*	Cereals (e.g., sorghum, rice, corn, wheat, barely), oil seeds (e.g., cotton seed, oilseed rape, sunflower seed), nuts (e.g., peanuts, groundnut, pistachio), spices (e.g., turmeric, black and red pepper, ginger), meats, dairy products, fruit juices, dried fruits, eggs, and feeds and foods derived from these products	[8,11,33-35]
Aflatoxin G2	*A. flavus*[1], *A. aflatoxiformans, A. austwickii, A. cerealis, A. arachidicola, A. minisclerotigenes, A. mottae, A. luteovirescens, A. nomius, A. novoparasiticus, A. parasiticus, A. pipericola, A. pseudocaelatus, A. pseudonomius, A. sergii, and A. transmontanensis*	Same as aflatoxin G1	[8,11,33-35]
Aflatoxin G2[a]	Hydroxylated metabolite of aflatoxin G1 obtained by catalytic addition of water to the double bond of the	NA	[33,38-39]

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	terminal furan under acidic conditions in the liver, the stomach or soil (no evidence for the involvement of specific enzymes). Naturally produced by *A. flavus*		
Aflatoxin GM1	Hydroxylated metabolite of aflatoxin G1 by MFO in the liver of mammals Produced in vitro by *A. parasiticus* fed aspertoxin as a precursor Naturally produced by *A. flavus*	Milk and dairy products	[41-42,55]
Aflatoxin GM2	Hydroxylated derivative of aflatoxin G2 by MFO in the liver of mammals Produced in vitro by A. parasiticus from dihydro-O-methylsterigmatocystin (DHOMST) Naturally produced by *A. flavus* and *A. parasiticus* and yeast	Milk and dairy products	[41-42]
Aflatoxin GM2[a]	Metabolite of aflatoxin GM1in the liver of mammals Hydration of the terminal furan ring of aflatoxin M1 in dilute acid to yield an hemiketal in vitro in liver homogenates	Milk and dairy products	[42]
Parasiticol (aflatoxin B3)	*A metabolite of aflatoxin G1 from the biodegradation (hydrolysis and decarboxylation reactions) in A. flavus, Rhizopus stolonifer, Rhizopus arrhizus, and Rhizopus oryzae*	Idem as aflatoxins B1 and G1	[11,55-58]
Others			
Parasiticol (aflatoxin B3)	A metabolite of aflatoxin G1 from the biodegradation (hydrolysis and decarboxylation reactions) in *A. flavus, Rhizopus stolonifer, Rhizopus arrhizus,* and *Rhizopus oryzae* Naturally produced by *A. parasiticus, A. flavus, A. mottae, A. nomius, and A. novoparasiticus*	Idem as aflatoxins B1 and G1	[11,55-58]
Aspertoxin[b]	*A. flavus and A. parasiticus*	Mainly vegetal products prone to contamination with *A. flavus* and A. parasiticus; not considered to be relevant to food products of animal origin	[41,59]

[a]Not a typical producer of G-types of aflatoxins, but some strains were reported to produce them in addition to B1 and B2 [11]; [b]Usually considered as a sperate mycotoxin produced by *A. flavus* because of structural differences with the difurocoumarin structure that characterizes the aflatoxins. *Abbreviations:* NA: Not available.

Table 2: Key properties of aflatoxins and their metabolites. Data compiled from PubChem of the National Center for Biotechnology Information (https://pubchem.ncbi.nlm.nih.gov) and ChemSpider of the Royal Society of Chemistry (http://www.chemspider.com) databases, unless references are indicated beside the data.

Aflatoxin	MW (g/mol)	Formula	Melting Point (°C)[a]	Toxicity			Adverse health effects[b]
				LD_{50} (mg/kg bw)	Test organism	Route	
Aflatoxin B1	312.063	$C_{17}H_{12}O_6$	268.5	0.24-60 [60] 3.0	Various animals and chick embryo Human	Oral, intraperitoneal or injection (chick embryo) In vitro experiments	Hepatotoxicity, genotoxicity, carcinogenicity, immuno-toxicity, teratogenicity
Aflatoxin B2	314.079	$C_{17}H_{14}O_6$	286-289 [60]	1.7	Duck	Oral	Week mutagenicity, hepatotoxicity, and carcinogenicity [48]
Aflatoxin $B2_a$	330.074	$C_{17}H_{14}O_7$	240 [60]	>400 µg showed a weak toxicity [61-62]	Ducklings	Oral	Low toxicity (200-fold less than B1) [37,62]
Aflatoxin M1	328.058	$C_{17}H_{12}O_7$	297-299	0.32 1.5	Duck Rat	Unreported Oral	Hepatotoxicity, nephrotoxicity, carcinogenicity
Aflatoxin M2	330.074	$C_{17}H_{14}O_7$	237-240	3.1 [42]	Ducklings [42]	Oral [42]	Same as M1 but to a lesser extent
Aflatoxin P1	298.048	$C_{16}H_{10}O_6$	240	>150 mg/kg > 190 ng/egg [60]	Mouse Chick embryo [60]	Intraperitoneal Injection [60]	Same as B1 but to a lesser extent
Aflatoxin Q1	328.058	$C_{17}H_{12}O_7$	250	207 ng/egg [63] NR	Chick embryo [63] Bacteria [48]	Injection [63] Ames' test [48]	Non-carcinogenic on fish [48] 50-fold less mutagenic than B1
Aflatoxicol (R_0)[c]	314.079	$C_{17}H_{14}O_6$	230-234 [60]	NA NR	NA Bacteria	NA Ames test	Hepatotoxicity, carcinogenicity and mutagenicity. Forms the same DNA-adduct as B1. Two to 18-fold less toxic than B1 [32,48,52,64-67]
Aflatoxicol M1[d]	330.074	$C_{17}H_{14}O_7$	215.31 (predicted)	NA NR	NA Bacteria [68]	NA Ames' test [68]	Low toxicity, mutagenicity, and carcinogenicity [48,56]
Aflatoxicol H1[d]	330.074	$C_{17}H_{14}O_7$	NA	Not toxic [54] NR	Chick embryo [54] Bacteria [54]	Injection 104] Ames' test [54]	Weekly toxic to inactive (A detoxified form of B1) [33]
Aflatoxin G1	328.058	$C_{17}H_{12}O_7$	244-246	0.8 [42]	Duckling	Oral	Hepatotoxicity, nephrotoxicity, Carcinogenicity (animals)

Aflatoxin G2	330.074	$C_{17}H_{14}O_7$	237-240 226-229	2.5 [42] Weekly mutagenic	Duckling S. typhimurium	Oral Ames' test	Low toxicity, no evidence for carcinogenicity in animals [33,48,69]
Aflatoxin G2a[d]	346.069	$C_{17}H_{14}O_8$	243.13 (Predicted)	NA	NA	NA	Low toxicity to inactive (a detoxified form of G1) [33,48]
Aflatoxin GM1	344.053	$C_{17}H_{12}O_8$	276	NA	NA	NA	NA
Aflatoxin GM2	346.069	$C_{17}H_{14}O_8$	270-272	NA	NA	NA	NA
Parasiticol	302.079	$C_{16}H_{14}O_6$	233.4-234.1[56]	05.0 to 10.0 µg/egg 50.0 µg/duck [56]	Chick embryo Duckling [56]	Injection Oral [56]	Lower toxicity than G1 Same acute toxicity as B1. No or weak carcinogenicity [56]
Aspertoxin	354.074	$C_{19}H_{14}O_7$	NA	0.7 µg/egg [70]	Chick embryo [70]	Injection [70]	Teratogenic on chicken. Same fatality rate in chick embryo as B1[70]

[a]Data collected from ChemSpider website (http://www.chemspider.com) unless indicated by an imbedded citation; [b]In the latest classification of mycotoxins, the IARC stated that there is "sufficient evidence" for the carcinogenicity of aflatoxins B1, G1, and M1 in experimental animals, but there is "limited evidence" or "insufficient evidence" in experimental animals for the carcinogenicity of aflatoxins B2 and G2, respectively; however, in view of mechanistic studies showing the ability of the major aflatoxins (B1, G1, B2, G2, M1) to form DNA adducts as a first step in genotoxicity, they were classified in group 1 carcinogens [69]; [c]Usually designated as the aflatoxin B1 reservoir, as it readily converts back to B1 by action of a dehydrogenase; [d]Mutagenicity induced in *Salmonella typhimurium* is <1% that of aflatoxin B1 taken as a reference [48]. *Abbreviations:* NA: Not available; NR: Not relevant; bw: Body weight.

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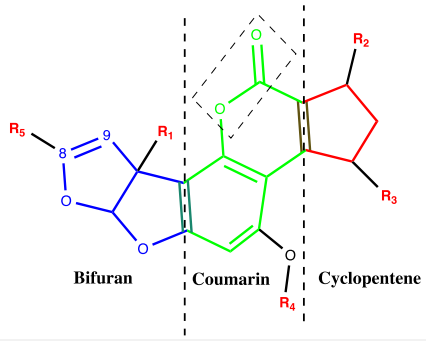
2.3 *Structural Diversity of Aflatoxins*

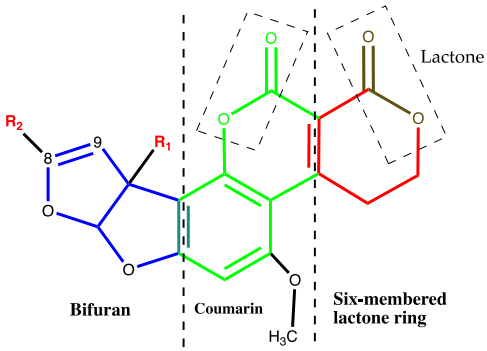
Structurally, aflatoxins are difuranocournarins/difurocoumarins synthesized via the polyketide pathway, and they consist of a coumarin nucleus (Figure 1 A and B, in green in the middle) to which are attached a difuran moiety in one side (Figure 1A, left in blue) and either a pentene ring (Figure 1 A, in red on the left) or a six-membered lactone ring in the other side (Figure 1B, red on the right). On this basis, aflatoxins fall into two main groups: (i) Difurocoumarocyclopentenone comprised typically of aflatoxin B series and derivatives (Table 1 and Figure 1A), and (ii) Difurocoumarolactone with the aflatoxin G series as the main representatives, typically including AFG1, AFG2, AFGM1, AFGM2, and $AFG2_a$ (Table 1 and Figure 1 B). Parasiticol (also designated aflatoxin B3) is usually considered as a member of the latter group despite the lack of the characteristic six-membered lactone ring (Figure 1 C, right) [57]. There also is a question as to whether or not aspertoxin is an aflatoxin due to its bifuroxhanthone structure that does not relate to members of either one of the difurocoumarin groups (Figure 1 C, left). This mycotoxin, which is structurally related to sterigmatocystin (an intermediate metabolite of aflatoxins B1 and G1) [31] can also be a precursor of aflatoxin GM1 [41], which may explain the raison for some authors to consider it as a member of the difurocoumarolactone group [71]. Contrary to other aflatoxins, aspertoxin has received the least attention despite its demonstrated toxicity in chicken embryos where it causes malformations, generalized oedema, loss of muscle tone, and haemorrhage from the umbilical vessels leading to death [70]. It is worth mentioning that aflatoxins with saturated (AFG2, AFGM2, and AFM2) or hydrated ($AFB2_a$, $AFG2_a$, $AFM2_a$, $AFQ2_a$, $AFG2_a$, $AFGM2_a$) terminal furan ring are the least toxic, indicating the crucial role that the $C^8=C^9$ double bond of this furan moiety plays in the toxicity of aflatoxins [48].

A: Difurocoumarocyclopentenone aflatoxins



Aflatoxin	R_1	R_2	R_3	R_4	R_5	C_8-C_9 bond
B1	H	=O	H	CH_3	H	Unsaturated
B2	H	=O	H	CH_3	H	Saturated
$B2_a$	H	=O	H	CH_3	OH	Saturated
M1	OH	=O	H	CH_3	H	Unsaturated
M2	OH	=O	H	CH_3	H	Saturated
$M2_a$	OH	=O	H	CH_3	OH	Saturated
P1	H	=O	H	H	H	unsaturated
Q1	H	=O	OH	CH_3	H	Unsaturated
$Q2_a$	H	=O	OH	CH3	OH	Saturated
Aflatoxicol B	H	OH	H	CH_3	H	Unsaturated
Aflatoxicol M1	OH	OH	H	CH_3	H	Unsaturated
Aflatoxicol H1	H	OH	OH	CH_3	H	Unsaturated

B: Difurocoumarolactone aflatoxins



Aflatoxin	R_1	R_2	C_8-C_9 bond
G1	H	H	Unsaturated
G2	H	H	Saturated
$G2_a$	H	OH	Saturated
GM1	OH	H	Unsaturated
GM2	OH	H	Saturated
$GM2_a$	H	OH	Saturated

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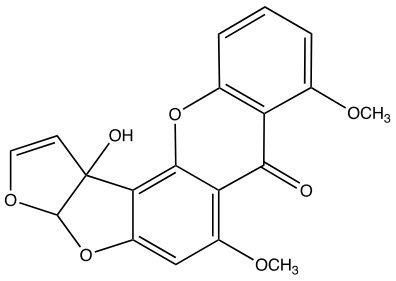
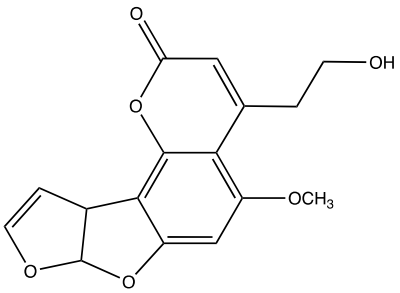
C : Other aflatoxins



Aspertoxin



Parasiticol

Figure 1: Diversity of chemical structures of aflatoxins in the difurocoumarocyclopentenone (A) and the difurocoumarolactone (B) groups. Aspertoxin, a difuranoxanthane, and parasiticol, lacking the lactone ring of its parent aflatoxin G1, are occasionally considered as standalone mycotoxins (C).

3 Aflatoxin Production and Incidence in Crops and Feeds

3.1 Crop Contamination

As discussed above, aflatoxin-producing moulds, particularly *A. flavus* and *A. parasiticus*, are frequent contaminants of crops where they grow and excrete aflatoxins, which can in turn be found in foods and feeds at high levels making them unfit for consumption. The USFDA considered unavoidable the contamination of agricultural products with aflatoxins that can, at best, be kept at the lowest practical levels to minimize the exposure of humans and animals [72]. However, despite the widespread of aflatoxins throughout the world, their prevalence in foods and feeds is higher in some regions than in others depending on the pedoclimatic conditions, the agricultural practices, the cultivars grown, the mechanical and insect damage of crops, and the awareness of the harmful effects of food-borne toxins on the productivity and safety of produce [73-74]. In addition to inherent traits that influence the toxigenicity of moulds, such as the species, the strain, the morphotype, and the competitiveness within the microbiome [10,20,75-77], the level of development of the country; the availability and degree of enforcement of pertaining regulations also account for the extent of food and feed contamination with aflatoxins [78-79]. Considering these factors, the highest incidence has classically been recorded in Sub-Sahara African and Southeast Asian countries, owing primarily to the favourable climatic conditions, and then to the low development status and the lack of public awareness of the risk these toxins pose to human and animal health. Table 3 presents the mean annual temperatures and rainfalls in selected countries among the most notorious for the high incidence of aflatoxins in their foods and feeds. Although in each of these countries there are different AEZ according to the definition of Köppen and Geiger [80], the hot, humid tropical and subtropical climates are predominating and provide ideal conditions for aflatoxin contaminations [74]. The mean annual temperatures in these countries vary between 22 and 29°C and the mean annual rainfalls are generally higher than 700 mm. Under such conditions, aflatoxigenic molds grow well and produce significant amounts of aflatoxins, especially when the water activity (a_w) of the produce falls within the range of 0.90 to 0.99 (Table 4). This may be the case if the crop is harvested before its moisture content is low enough (<15%) or stored in an environment with high relative humidity (RH) and poor aeration [21,74,81]. Other growth parameters, such as the pH and nature of the soil, the availability of carbohydrates, nitrogen, phosphates, zinc, and various trace metals also affect the production of aflatoxins [82], but none of which appears to be a limiting factor in the countries considered. These favourable environmental conditions are enhanced by the vulnerability of the prevailing agricultural systems. Farming activities are essentially managed for subsistence by smallholders facing technical and socio-economic challenges that hamper any efforts to restrain aflatoxin contamination [83-85]. Moreover, the staple crops grown, such as peanut, maize, sorghum, rice, sunflower, and cottonseed are good substrates for aflatoxin production [86-87].

Since the late 1970s, intensive research has been conducted to assess the extent of aflatoxin contamination of different foods and feeds in these regions, and the results were used by the IARC working groups to relate aflatoxin dietary intake to liver cancer. Published data on the contamination of staple crops with aflatoxins in selected countries from Sub-Saharan Africa (West and South-East regions) and South-Eastern Asia are compiled in Tables 7 and 8, respectively. The tables show that peanut/groundnut and maize are the most highly and frequently contaminated products, whereas millet and rice are generally less contaminated, although not always with safe levels. The climates that predominate in AEZs where the highest aflatoxin levels were recorded are warm arid and semi-arid, tropical, or subtropical or irrigated desert [82] (see also, Tables 7 and 8). In addition to the climate type, an annual mean rainfall around 700 mm is an additional factor that favours aflatoxin contamination [21]. A positive relationship between the rainfall and aflatoxin concentration was demonstrated in sorghum grown in four different AEZs in Nigeria, where the contamination with aflatoxin B1 was highest in the zone with rainfalls exceeding 1400 mm [88]. Nonetheless, aflatoxin contamination of peanut and maize was reported to be maximal at an average

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annual rainfall between 600 and 700 mm and decrease exponentially thereafter [21]. This may partly explain the consistently high aflatoxin levels and incidence in foods (Table 5) and feeds (Table 7) in Kenya where the mean annual rainfall is about 670 mm (Table 4). According to a survey on aflatoxin contamination of maize conducted in the country during the period 2006-2009, only 17% and 5% of the production is fit for human and animal consumption, respectively [89]. However, due to food shortage and lack of awareness of the inherent health risks, these foods are eaten by local populations, which explains why this country has been repeatedly and severely afflicted by aflatoxicosis outbreaks [90-91]. Recent data suggest that that the situation did not improve since then and the dietary exposure to aflatoxins remains too high. The probable daily intake (PDI) of aflatoxin B1 in the country, via maize only, was recently estimated to vary between 0.07–60,612.00 ng/kg bw/day, with an average of 312.4 ng/kg bw/day) [92], which is alarming compared with an average of 10 to 200 ng/kg/day for the rest of the world [93], and with the conservative tolerable daily intake (TDI) of 0.11 to 0.19 ng/kg bw/day [94]. Incidentally, this country also ranks among the countries with the highest prevalence of oesophageal cancer, which was associated with aflatoxin intake as a risk factor [18,92,95]. Conversely, a recent survey on aflatoxin contamination of maize grown in eight different AEZs in Uganda revealed that the highest levels (a maximum of 3760 µg/kg) and an average of 66.5 µg/kg) were recorded in the zones with high rainfalls (1200->1400 mm); the percentage of samples exceeding the national regulatory standards of 10 µg/kg reached 22.2% [96].

Table 3: Climatic conditions in countries reputed for their vulnerability to aflatoxin contamination. Data are mean values for the period of 1901-2016. Sources: https://climateknowledgeportal.worldbank.org, https://www.climatedata.eu, and https://en.climate-data.org.

Region/ Country	Annual temperature (°C)			Mean annual rainfall (mm)	Predominating climate types[a]
	Min	Max	Mean		
Sub-Saharan Africa					
Benin	25.3	30.3	27.5	1059	Tropical savanna (Aw)
Cameroun	23.4	26.7	24.8	1614	Tropical savanna (Aw)
Ghana	25.3	29.5	27.3	1190	Tropical savanna (Aw)
Kenya	22.6	25.9	24.3	669	Tropical savanna (Aw)
Mali	21.2	33.4	28.3	333	Tropical savanna (Aw)
Nigeria	18.5	32.4	25.4	881	Tropical savanna (Aw)
Tanzania	19.9	23.5	22.2	998	Tropical savanna (Aw)
Togo	25.0	29.5	27.0	1170	Tropical savanna (Aw)
Uganda	21.3	23.6	22.4	1200	Tropical savanna (Aw)
Zambia	17.2	25.0	22.0	976	Humid subtropical (Cwa)
South Africa	14.6	25.9	20.3	779	Temperate oceanic (Cfb)
Southeast Asia					
India	17.0	30.0	24.1	1057	Tropical savanna (Aw)
Indonesia	22.8	30.2	28.9	2859	Tropical rainforest (Af)
Malaysia	24.9	25.9	25.4	3059	Tropical rainforest (Af)
Philippines	24.3	27.0	25.5	2471	Tropical rainforest (Af)
Thailand	23.0	28.9	26.3	1553	Tropical savanna (Aw)

[a]In the same country, there are generally more than one climate type depending on the geographical region, which is defined as agroecological zone (AEZ) according the classification of Köppen-Geiger (http://koeppen-geiger.vu-wien.ac.at), where the first letter refers to the climate

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296 type (A: Tropical; B: Arid; C: Warm temperate), the second letter refers to the precipitation (w:
297 Winter dry; S: Steppe; f: Fully humid; m: Monsoonal), and the third letter refers to the temperature
298 (h: hot arid; a: Hot summer; b: Warm summer).

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Table 4: Minimum (Min), maximum (Max) and optimum (Opt) values of Temperature (°C) and water activity (a_w) for the growth and aflatoxin production by *Aspergillus flavus* and *Aspergillus parasiticus* in selected grains and in laboratory media.

Substrate/ Parameter	Growth						Aflatoxin production						References
	A. flavus			*A. parasiticus*			*A. flavus*			*A. parasiticus*			
	Max	Min	Opt	Max	Min	Opt	Max	Min	Opt	Max	Min	Opt	
Wheat/													
Temperature	>42.5	15	35	-	-	-	42.5	15.0	25	-	-	-	[97]
a_w	>0.95	0.80	0.95	-	-	-	0.95	0.85	0.93	-	-	-	
Nyjer seeds[a]/													
Temperature	NS	NS	27	NS	NS	27	NS	20	27.0	NS	20	27.0	[98]
a_w	NS	0.82	0.98	NS	0.82	0.94	NS	0.86	0.90	NS	0.86	0.98	
Sorghum/													
Temperature	NS	15	37	-	-	-	NS	15	37	-	-	-	[99]
a_w	NS	<0.91	0.97	-	-	-	NS	0.94	0.97	-	-	-	
Rice/													
Temperature	42	20	33	-	-	-	37	<20	35	-	-	-	[100]
a_w	0.99	0.80	0.90	-	-	-	0.99	0.85	0.96	-	-	-	
Sabouraud/													
Temperature	NS	0.90	0.99	-	-	-	NS	0.90	0.99	-	-	-	[101]
a_w	NS	15	NS	-	-	-	NS	15	NS	-	-	-	
Malt Extract-Sucrose/													
Temperature	-	-	-	42	15	35	-	-	-	40[b]	17[b]	37[b]	[102]
										37[c]	17[c]	20[c]	
	NS	12	37	NS	13	32	37	12	31	NS	10-13	24	[103-104]
	42	15	30-35	-	-	-	-	-	-	35	<20	25	[77]
a_w	-	-	-	NS	0.90	0.99	-	-	-	NS	0.90[b]	0.93[b]	[102]
										NS	0.90[c]	0.99[c]	
	NS	0.80	0.99	NS	0.83	>0.99	NS	0.85	0.99	NS	0.91	0.99	[103-104]
	-	-	-	-	-	-	-	-	-	0.99	0.85	35	[77]

*Scientific name *Guizotia abyssinica*, also called thistle or Niger seeds extensively used in Sub-Sharan region to extract oil, [b]For aflatoxin B1 production, [c]For aflatoxin G1 production.

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It should be pointed out, however, that despite the well-established impact of the climate type on the extent of crop contamination with aflatoxins, no direct correlation between aflatoxin levels and the AEZs has been established. On the contrary, in a survey on aflatoxin contamination of maize and groundnut samples collected from 27 districts of three different AEZs in Zambia no such correlation could be established [21]. The levels of aflatoxins in a product within the same AEZ were shown to vary greatly depending on the rainfall and temperature variations from one year to another, in addition to the experimental design and the sampling point [105]. In fact, there is a multiplicity of factors that interfere with the effect of the climate type at different production stages from plant development to crop storage [18,74]. It is increasingly recognized that management systems with rigorous implementation of the good agricultural practices (GAPs) and farmer trainings are critical measures to mitigate the incidence of aflatoxins in agricultural products regardless of the climate type [18,79,92,106-108]. The implementation of such measures in South Africa in the framework of a project on the "Adaptation of agricultural practices to climate change in Sub-Saharan Africa (CAADP)" aimed at "good agricultural adaptation practices" [109], appears to have been successful in controlling aflatoxin contamination. The most recent survey in the country revealed very low incidence and contamination levels of AFB1 in peanut and wheat samples collected from all the country regions during the period of 20124 and 2018 [110]. Nonetheless, climatic shifts and occurrence of drought periods followed by heavy rains that occur in the region, remain a challenging issue which may counteract these measures and induce a rebound in the levels of aflatoxin contamination [74,111]. Indeed, the major documented aflatoxicosis outbreaks were reported to coincide with drought periods followed by unseasonal heavy rain or in regions with frequent and unpredictable temperature and rainfall shifts due to the so-called El Niño-Southern Oscillation (ENSO) phenomenon [112]. A first outbreak in India in 1974 was caused by the consumption of contaminated maize in two chronically drought-stricken districts which received unseasonal rain while the maize was mature and ready to harvest [113]. In Kenya, the major aflatoxicosis outbreak of the year 2004 was preceded by a severe drought followed by heavy rains during the harvest period of the maize implicated in the outbreak [90], as discussed below (paragraph 4.1). The same country has experienced another drought in 2009, which was also followed by a significant increase in maize contamination with aflatoxins resulting in the condemnation of 10% of the production in the following year [114-115]. The water stress caused by drought weakens the plant defence and increases its susceptibility to mould infection and aflatoxin production [105], which may be further enhanced if the crop is harvest in the rainy season. A recent survey on the contamination of food products of West Africa Sub-Saharan countries revealed that aflatoxin contamination of crop samples collected during the rainy season was significantly higher than those collected during the dry season [87]. Similar observation could be made from a comprehensive survey on aflatoxin contamination of various foods in Thailand for the period of 1969-1970 [116-117] (see also Table 6).This highlights the primary impact of temporal distribution of rainfall, rather than its quantity throughout the year, on the extent of aflatoxin contamination. Harvesting during the rainy season yields crops that have not yet reached low-enough moisture content to resist mould colonization. Yet, moisture content of the crop at harvest is not the only explanation of the phenomenon, as aflatoxin contamination was shown to be highest when high rainfall occurred during the pre-flowering stage and lowest during the flowering and post-flowering stages, not including harvest [76]. This was explained by the healthy status of the plant in the latter stages, which should be accompanied by a high vegetation cover, which increases the plant's resistance to mould invasion and aflatoxin production [76].

Inappropriate storage conditions also play a major role in the increase of aflatoxin contamination; and increased aflatoxin levels from field to storage structures is well documented. For example, a 26-fold increase in aflatoxin concentration was observed in sorghum grown in Niger state (Nigeria) from field to storage in traditional mud-built barns [88]. Also, the maximum aflatoxin concentration in maize increased from 26.5 µg/kg at harvest to 1460 µg/kg after 4 months of storage at the farmers' household in South-eastern Nigeria [118]. Moreover, Villers [114] quoted that aflatoxin concentration increased by 200 times in peanut after 2 months of storage under conventional conditions in Mali and by 300 times in maize after 3

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351 months of storage in traditional facilities in Uganda. Such results were corroborated on bambara nut (*Vigna*
352 *subterranean*, L), groundnut, maize, sunflower, and sorghum from different AEZs in Tanzania [108].
353 Strikingly high aflatoxin levels were recorded in commercial peanut samples collected from different
354 marketing structures in Kenya, with the highest levels, e.g., 32,328 µg/kg, being recorded in informal
355 market outlets and poorly designed stores of retailers and stockists [119], see also Tables 7 and 8. Under
356 experimental conditions, aflatoxin concentrations in both peanut and maize increased by more than 1000
357 times after one week of storage at 31°C and 100% relative humidity compared with safe levels in freshly
358 harvested crops [21].

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Table 5: Incidence (%) and concentrations (µg/kg) of aflatoxins in staple agricultural products of selected countries from Sub-Saharan Africa. Data are for total aflatoxins (B1+B2+G1+G2), unless otherwise stated in the footnotes.

Country	AEZ	Peanut /Groundnut		Maize		Millet		Sorghum		Sunflower		References
	(Climate type)[a]	Mean[b] (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	
Uganda	Kioga planes[c] (Am)	7.3-221 (2.5-450)	20-60	25.4-71 (4.5–180)	50-80	-	-	61-170 (4-26)	80-100	-	-	[86]
	Western (Aw)	7.0 (3-13)	25	75.2 (3.5-248)	95	-	-	-	-	-	-	
	Savannah (Aw)	8.8 (5.5-12)	30	26.56 (3.3-105)	100	-	-	11.5 (29–472)	100	-	-	
	Grasslands (Aw)	85.4 (2.5-175)	30	46.0 (3.1–510)	95	-	-	102.3 (28-227)	100	-	-	
	South-East (Af)	-	-	-	-	14.0 (NS-NS)	100	-	-	-	-	[120]
	Commercial[d]	181 (0-849)	82	-	-	-	-	-	-	-	-	[121]
Kenya	Tharaka-Nithi (Cbw)	-	-	24[e(R)] (<1-537)	885[e(R)]	11[e(R)] (<1.0-152)	64[e(R)]	1.2[e(R)] (<1-18)	33[e(R)]	-	-	[122]
		-	-	23.9[e(D)] (<1.0–775)	75.4[e(D)]	66[e(D)] (<1-1658)	69[e(D)]	1.5[e(D)] (<1.0 – 23.1)	85[e(D)]	-	-	
	Kisii (Af and Cfb)	NS (1.6-591)	100	-	-	-	-	-	-	-	-	[123]

	-	-	4.0[e(R)] (<1-103)	46.8[e(R)]	0.1[e(R)] (<1-3.0)	21[e(R)]	0.9[e(R)] (<1-16.4)	11[e(R)]	-	-	[122]
	-	-	8.9[e(D)] (<1.0–372)	78[e(D)]	0.5[e(D)] (<1-2.9)	77[e(D)]	-	-	-	-	
	-	-	28.5[e] (<1.0–559)	76.3	-	-	-	-	-	-	[92]
Migori (Am)	-	-	12.7[e] (0.98–121)	56	-	-	-	-	-	-	
Bungoma (Cfb)	-	-	7.9[e(R)] (<1-218)	81[e(R)]	0.6[e(R)] (0–2.9)	98[e(R)]	3.5[e(R)] (<1-92)	97[e(R)]	-	-	[122]
	-		3.5[e(D)] (<1.0–39.3)	72[e(D)]	0.9[e(D)] (<1.0 – 13.8)	75[e(D)]	0.9[e(D)] (<1.0-12.3)	84[e(D)]			
Isiolo (Aw)	-	-	9.6[e(R)] (<1-121)	98[e(R)]	-	-	3.8[e(R)] (<1-12.8)	100[e(R)]	-	-	
	-	-	67.3[e(D)] (<1.0-1137)	50[e(D)]	-	-	2.0[e(D)] (<1.0–11.9)	57[e(D)]	-	-	
Kwale (As)	-	-	29[e(R)] (<1-394)	97[e(R)]	-	-	-	-	-	-	
	-	-	3.5[e(D)] (<1.0 – 19.2)	95[e(D)]	-	-	-	-	-	-	
Eldoret (Cfb)	1147 (NS-NS)	NS	-	-	-	-	-	-	1524 (NS-NS)	NS	[124]
Nandi (Aw)			1.3[f] (0–3.92)	-	-	-	-	-	-	-	[107]
	-	-	0.98 (0.1–5.3)	68	1.6 (0.14–11)	92	24.5 (0.15-210)	66	-	-	[125]

Makueni (Aw)	-	-	24.8 (0.1-279)	80	17.2 (0.4-231)	82	17.8 (0-265)	86	-	-	
Busia (Am)	NS (0.1-268)	97.1	-	-	-	-	-	-	-	-	[123]
	NS (>20-7525)	7.5	-	-	-	-	-	-	-	-	[18]
Homabay (Aw)	-	-	24.5[e] (0.98–722)	56	-	-	-	-	-	-	[92]
Kitale (Cfb)	-	-	9.7 (0-72)	70	-	-	-	-	-	-	[126]
Nakuru (Cfb)	-	-	4.2 (0-13)	97	-	-	-	-	-	-	
Makueni-Kitui (Aw, BSh)	-	-	9. 1[g(Gm)] (0–48,000)	35[h]	-	-	-	-	-	-	[127]
Korogocho (Cwb)	-	-	6.7 (0-89)	NS	-	-	8.1 (0.2-194)	NS	-	-	[128]
Dagoretti (Cfb)	-	-	3.0 (0-20)	NS	-	-	2.6 (0.1-15)	NS	-	-	
Makueni (BSh, Aw, Cwb)	-	-	52.9[g(Gm)] (<1-5400)	59[h]	-	-	-	-	-	-	[90]
	-	-	39[e] (0.01–1455)	68	-	-	-	-	-	-	[92]
	-	-	24.8 (0.05–279)	80	17.2 (0.4–231)	82	17.8 (0.04–265)	86	-	-	[129]
Embu (Aw)	-	-	196.3[e] (0.95–9092)	64	-	-	-	-	-	-	[92]
Kitui (BSh and Aw)	-	-	0.7 (0-13)	97	-	-	-	-	-	-	[126]

		-	-	35.3[g(Gm)] (<1-25,000)	45[h]	-	-	-	-			[90]
	Machakos (Cwb)	-	-	17.8[g(Gm)] (<1-3800)	52[h]	-	-	-	-	-	-	
		-	-	11[5] (1.3–71)	61	-	-	-	-	-	-	[92]
	Thika (Cwb)	-	-	7.52[g(Gm)] (<1.0-46,400)	25[h]	-	-	-	-	-	-	[90]
	Commercial[4]	NS (>4.0-32,328)	49	-	-	-	-	-	-	-	-	[119]
Tanzania	Long, Babati (Cwb)	-	-	2.6 (2.1-3.6)	17	-	-	-	-	-	-	[130]
	Sabilo, Babati (Cwb)	-	-	3.32 (2.2-26)	28	-	-	-	-	-	-	
	Seloto, Babati (Cwb)	-	-	2.62 (2.1-4.0)	13	-	-	-	-	-	-	
	Tabora (Aw)	-	-	NS (5-158)	37	-	-	-	-	-	-	[131]
	Kilimanjaro (Cwb)	-	-	NS (1.0-80)	20	-	-	-	-	-	-	
	Ruvuma (Aw)	-	-	NS (7-26)	6	-	-	-	-	-	-	
	Iringa (Cwb)	-	-	NS (13-58)	7	-	-	-	-	-	-	
	Kilosa (Aw)	-	-	106 (3.0-1081)	18	-	-	-	-	-	-	[132]
	Hanang' (Csb)	-	-	4.0 (3.0-5.0)	8	-	-	-	-	-	-	

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	Rungwe (Cwb)	-	-	5 (2-8)	4	-	-	-	-	-	-	
	Chitego (Aw)	21.9 (0-56)	NS	-	-	-	-	9.1 (0-62.5)	NS	19.0 (0-605)	NS	
	Laikala (Bsh)	84.9 (0-427)	NS	0.76 (0-1.2)	NS	-	-	2.7 (0-29.8)	NS	61.1 (0-489)	NS	
	Mlali (Bsh)	85.4[5] (0-298)	NS	2.8[5] (0-22)	NS	-	-	25.7[5] (0-70)	NS	4.9[5] (0-44)	NS	[108]
	Moleti (Bsh)	377.3[5] (0-3297)	NS	4.2[5] (0-43)	NS	-	-	9.4[5] (0-73.9)	NS	100[5] (0-425)	NS	
	Njoro (Csb)	289.7[5] (0-1179)	NS	2.5[5] (0-29.2)	NS	-	-	93.3[5] (0-138.7)	NS	82.0[5] (0-295)	NS	
	Babati (Cwb)	-	-	-	-	-	-	-	-	46.8[i] (1.8-162)	83	
	Singida (BSh)	-	-	-	-	-	-	-	-	45.8[i] (1.4-262)	83	
	Dodoma (BSh)	-	-	-	-	-	-	-	-	59.6[i] (1.7-281)	71	[133]
	Mbeya (Cfa)	-	-	-	-	-	-	-	-	21[i] (1.4-174)	89	
	Morogoro (Aw)	-	-	-	-	-	-	-	-	119[i] (2.8-663)	50	
Zambia	Chipata (Aw)	451 (>1-4000)	NS	-	-	-	-	-	-	-	-	
	Petauke (Cwa)	4.34 (>1-10)	NS	-	-	-	-	-	-	-	-	
	Ndola (Cwa)	242 (>1-1600)	NS	-	-	-	-	-	-	-	-	[4]
	Katete (Cwa)	13.6 (>1-74)	NS	-	-	-	-	-	-	-	-	

	Kitwe (Cwa)	499 (>1-11,100)	NS	-	-	-	-	-	-	-	-	
	Kabwe (Cwa)	21.4 (>1-145)	NS	-	-	-	-	-	-	-	-	
	Southern area[j] (BSh, Cwa)	22.0 (3.9-621)[k]	100[l]	12.0 (3.9-621)[k]	73[l]	-	-	-	-	-	-	[21]
	Central area[m] (Cwa)	90 (0-3420)[k]	51[l]	11.0 (0-3420)[k]	42[l]	-	-	-	-	-	-	
South Africa	Northen area[n] (Cwb)	6.0 (0-1416)[k]	27[l]	25 (0-1416)[k]	22[l]	-	-	-	-	-	-	[134]
	Limpopo (BSh)	-	-	48[e] (0-133)	20	-	-	-	-	-	-	
	Mpumalanga (Cfb)	-	-	1 (1-2)[e]	6.5	-	-	-	-	-	-	[134]
	Commercial[d]	14 (0-74)	90	-	-	-	-	-	-	-	-	[135]
	All regions	ND	0	-	-	-	-	-	-	-	-	[110]
Nigeria	Kano (BSh)	6.0[o(LB)] (<0.1-97)	26	-	-	-	-	-	-	-	-	[87]
	Lagos (Aw)	-	-	0.6[o(LB)] (0-5)	10	-	-	-	-	-	-	
	Sokoto (BSh)	96.0 (1-415)	-	-	-	-	-	-	-	-	-	[136]
	Isa (BSh)	64.0 (2.0-317)	-	-	-	-	-	-	-	-	-	

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	Tambuwal (BSh)	92.9 (0.9-646)	-	-	-	-	-	-	-	-	-	
	Ogun (Aw)	-	-	300 (NS-NS)	NS	34.3 (NS-NS)	NS	221 (NS-NS)	NS	-	-	[137]
	Lagos (Aw)	-	-	603 (NS-NS)	NS	120.5 (NS-NS)	NS	1245 (NS-NS)	NS	-	-	
	South-East (Am, Aw)	-	-	43(Gm) (2.7-1460)	87.5P	-	-	-	-	-	-	[118]
	Western states (Aw)	-	-	200 (25-770)	45	-	-	-	-	-	-	[138]
	Suleja and Tafa (Aw)	-	-	-	-	-	-	225[5](ML) (0-728)	64	-	-	[88]
	Borgu and Magama (Aw)	-	-	-	-	-	-	210[5](ML) (0-712)	55	-	-	
	Minna Mokowa (Aw)	-	-	-	-	-	-	165[5](ML) (0-721)	57	-	-	
	Mariga-Rafi-Wushishi (Aw)	-	-	-	-	-	-	198e(ML) (0-1164)	45	-	-	
Cameroun	South-West (Am, Af)	26e (6.0–125)	NS	100e (6-645)	NS	-	-	-	-	-	-	[139]
	South-East (Am)	22e (6.0–77)	NS	96e (6-216)	NS	-	-	-	-	-	-	
	Western highland (Aw)	22e (6.0–110)	NS	47e (6-210)	NS	-	-	-	-	-	-	

Ghana	Ashanti (Aw, HF)	2.2 (0-17)	NS	6 (0–135)	NS	-	-	-	-	-	-	[140]
	Brong Ahafo (Aw, HF)	5.5 (0-54)	NS	0.6 (0-9)	NS	-	-	-	-	-	-	
	Volta (Aw, HF)	42.4 (0-387)	NS	9.0 (0-83)	NS	-	-	-	-	-	-	
	Brong Ahafo (Aw, DS)	145.6 (0-1999)	NS	16.8 (0–226)	NS	-	-	-	-	-	-	
	Northern (Aw, DS)	78 (0-3868)	NS	15.9 (0–341)	NS	-	-	-	-	-	-	
Togo	Volta (Aw, DS)	0.3 (0-1.0)	NS	24.2 (0–157)	NS	-	-	-	-	-	-	[141]
	Northern (Aw, SGS)	34.9 (0-168)	NS	6.8 (0–59)	NS	-	-	-	-	-	-	
	Upper East (Aw, SGS)	0.3 (0-1.0)	NS	15.4 (0–82)	NS	-	-	-	-	-	-	[141]
	Upper West (Aw, SGS)	15.9 (0–181)	NS	16.4 (0–190)	NS	-	-	-	-	-	-	
	Akomadan (Aw, FRT)	-	-	NS (0-112)	83	-	-	-	-	-	-	[142]
	Ejura (Aw, FRT)	-	-	NS (1-945)	100	-	-	-	-	-	-	
	Wenchi (Aw, SVT)	-	-	NS (0-23)	71	-	-	-	-	-	-	
	Fumesua (Aw, RFR)	-	-	NS (0-692)	78	-	-	-	-	-	-	
	Commercial[d]	-	-	38.7 (3-275)	42	-	-	14 (6-19)	25	-	-	[141]

Benin	Littoral (Aw)	7.6[o(LB)] (<0.1-105)	19	-	-	-	-	-	-	-	-	[87]
	Borgou (Aw)	-	-	1.6[o(LB)] (<0.1-20)	32	-	-	-	-	-	-	
Mali	Bamako (Aw)	9.4[o(LB)] (<0.1-246)	15	-	-	-	-	-	-	-	-	
	Sikasso (Aw)	2.2[o(LB)] (<0.1-43)	29	-	-	-	-	-	-	-	-	

*The type of climate (in the parenthesis) is defined according to Köppen-Geiger classification (http://koeppen-geiger.vu-wien.ac.at): Cfb: Warm temperate (C) fully humid (f) warm summer (b); Cwa: Warm temperate (C) winter dry (w) hot summer (a); Cwb: Warm temperate (C) winter dry (w) warm summer (b); Af: Tropical (A) fully humid (f); Aw: Tropical (A) winter dry (w); As: Tropical (A) steppe (s); Am: Tropical (A) monsoonal (m), Csb: Warm temperate (C) steppe (s) warm summer (b); BSh: Arid (B) steppe (S) hot (h), Cfa: Warm temperate (C) fully humid (f) hot summer (a), **Arithmetic mean as a default, geometric mean or median when followed by Gm or Md, respectively; [3]Different regions each has its own mean, minimum and maximum, and incidence values, [4]Commercial samples can be from different origins and, hence, their aflatoxin contents may reflect their origin and the storage conditions rather than the area where they are sold, [e]Data are relative to the occurrence of aflatoxin B1; either in the [(R)]rainy season or the [(D)]dry season; [f]Results field training for farmers with supervised application of the good agricultural practices; [g]Exceptionally high aflatoxin levels recorded in 2004 during a major aflatoxicosis in Kenia; [h]Percentage for samples containing more than 20 mg/kg of aflatoxins; [i]Samples collected from micro- and small-scale sunflower oil processors during the harvesting season of 2014; [i]Rainfall below 800 mm, high temperature (30°C); [k]The highest and lowest aflatoxin concentrations were not discriminated between peanut and maize samples by the authors; [l]Percentages were calculated for samples containing more than 4.0 µg/kg of aflatoxins; [m]High rainfall (900-1300 mm), moderate temperature (23-25°C); [n]High rainfall, cool temperature (16°C); [o]Total aflatoxins (AFB1+AFB2+AFG1=AFG2); [p]Pourcentage of samples contaminated with levels exceeding with more than 4 µg/kg after 4 months of storage. *Abbreviations and symbols*: AEZ: Argo-ecological zone; +ve; Positive samples (aflatoxin levels higher than the level of detection LOD unless specified otherwise); "-" no available data, 0 "zero": aflatoxin level below LOD; HF = Humid Forest, DS = Derived Savanna, and SGS = Southern Guinea Savanna. SVT=Savana Transition; RFR=Rain Forest; FRT=Forest Transition; ML: Mouldy samples (biased sampling procedure was used by the authors); LB: Lower bound (scenario where the concentration of non-detected analyte is zero and the concentration of detected but non-quantified analyte is the limit of detection).

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The situation of crop contamination with aflatoxins that prevails in Southeast Asia region is similar to that describe above for the African countries, with maize being the most highly and frequently contaminated in all of the countries listed in Table 6. Despite the vast area covered by the region, its climate types are less diversified than in Sub-Sahara African countries. The climate in most countries of the region is mainly tropical or subtropical with narrow mean annual temperature variations (21°C and 29°C), and a high annual rainfall (Table 4). The region is subject to monsoonal weather system producing marked rainy and dry seasons during the year, thereby providing favourable conditions for mould growth and aflatoxin production [143]. The predominating climate sub-types in the AEZs with high levels of aflatoxin contamination are Aw (Tropical savanna), Cwa (Humid subtropical), and Af (Tropical rainforest) (Table 6). Among these countries, India, Malaysia, and Thailand have experienced episodes of aflatoxicosis traced to the consumption of heavily contaminated foods with aflatoxins [113,144-145], consistent with high aflatoxin levels recorded in their staple crops, including peanut, maize, and sorghum. In contrast, rice that represents the primary staple food in this region, is the least contaminated according to the published data reviewed in this study (Table 6).

388 **Table 6**: Incidence and concentrations (µg/kg) of aflatoxin contamination of staple crops in selected countries from the Southeast Asian
389 region. Data are for total aflatoxins (B1+B2+G1+G2), unless otherwise stated in the footnotes

Country	AEZ (Climate type)[a]	Peanut /Groundnut		Maize		Rice		Sorghum		References
		Mean[b] (Range)	+ve (%)	Mean[b] (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	
India	20 states (Various)	-	-	-	-	NS (0.1-308)[c]	68	-	-	[146]
	Karnataka (BSh, Aw)	510.7[c] (NS-NS)	NS	67.3[c] (201-714)	100	-	-	882[c] (582-1250)	100	[147]
	Eastern region (Cwa, Aw)	-	-	<5[c(Md)] (0-120)	47[d]	-	-	-	-	[148]
	Western region (BSh)	-	-	15[c(Mm)] (0-333)	53[d]	-	-	-	-	
	North (BSh, Cwa)	-	-	30[c(Md)] (0-666)	69[d]	-	-	-	-	
	Southern region (Aw)	-	-	<5[c(Md)] (0-400)	21[d]	-	-	-	-	
	Mahashtra (BSh, Aw, Am)	-	-	-	-	-	-	NS (0.49-139)	82	[149]
	Rajasthan (BWh, BSh)	-	-	-	-	-	-	NS (0.1-15)	86	
	Tamil Nadu (Aw)	-	-	-	-	-	-	NS (0.01-264)	88	
	Punjab (Af)	-	-	-	-	NS (0->30.0)	91	-	-	[150]
Nepal	Eastern region (Cfa)	NS (54–1806)	34	NS (64–859)	32	-	-	-	-	[151]
The Philippines	NS	58 (0-885)	65	76 (0.0-1152)	95	-	-	-	-	[152]
	Iloco (Aw)	-	-	22 (NS-30)	NS	-	-	-	-	[153]
		-	-	39 (NS-1215)	NS	-	-	-	-	
	South Catabato (Af, Aw)	-	-	68.0 (NS-178)	NS	-	-	-	-	

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	Commercial	-	-	-	-	1.5 (0-8.7)	95	-	-	[154]
Thailand	Northeast (Aw)	-	-	-	-	0.8 0-13.4	63	-	-	[155]
	Central region (Aw)	-	-	-	-	1.7 (0-26.6)	53	-	-	
	Singburi (Aw)	245(R) (NS-NS)	56	-	-	-	-	-	-	[116]
		139(D) (NS-NS)	41	-	-	-	-	-	-	
		28(H) (NS-NS)	91	-	-	-	-	-	-	
	Ratburi (Aw)	329(R) (NS-NS)	63	-	-	-	-	-	-	
		71(D) (NS-NS)	63	-	-	-	-	-	-	
		99(H) (NS-NS)	72	-	-	-	-	-	-	
	Songkhla (Am)	207(R) (NS-NS)	47	-	-	-	-	-	-	
		96(D) (NS-NS)	70	-	-	-	-	-	-	
		62(H) (NS-NS)	68	-	-	-	-	-	-	
	Whole country	1563(R) (0-12,256)	NS	-	-	-	-	-	-	[117]
		1811(D) (0-9500)	NS	-	-	-	-	-	-	
		1203(H) (0-7660)	NS	-	-	-	-	-	-	
	Commercial	1530 (0-12,256)	49	400 (0-2730)	39	67 (0-248)	2	-	-	
		47 (0-304)	80	196 (0-750)	NS	-	-	-	-	[156]

		31.5 (2.2-171)	NS	-	-	-	-	-	-	[79]
Malaysia	Penang Island (Af)	NS (17-711)	43	-	-	-	-	-	-	[157]
		-	-	-	-	NS (1.1-5.2)	NS	-	-	[158]
	NS	NS (20-1000)	16	-	-	-	-	-	-	[159]
	Commercial	11.3 (0-103)	79	-	-	-	-	-	-	[160]
	Commercial	-	-	-	-	NS (0.15-4.4)	25	-	-	[161]
	Commercial	4.3[c] (1.5-15.3)	85	-	-	1.75[c] (0.7-3.8)	70	-	-	[162]
Indonesia	East Java (Aw)	-	-	149 (NS-390)	100	-	-	-	-	[159]
	Lampung (Af)	-	-	144 (0-350)	92	-	-	-	-	
	Commercial	-	-	464 (NS-490)	100	-	-	-	-	

Captions and abbreviations are as defined in the footnotes of Tables 7, unless otherwise specified herein ; [b]Arithmetic mean as a default value or geometric mean[(Gm)] or median[(Md)]; [c]Percentage of samples containing more than 5.0 µg/kg of aflatoxin; [(R)]Rainy season; [(H)]Hot season; [(D)]Dry season.

3.2 *Feed Contamination*

Crop residues and by-products from grain mills and/or oil extraction factories are often used as animal feeds. In developing countries, mouldy cereals and nuts of low grades are generally sorted to be fed to animals either directly or as ingredients in manufactured feeds [88,134,163]. Therefore, it is reasonable to anticipate that such feeds are more likely to be highly contaminated with aflatoxins than their counterpart crops destined to human consumption. This appears to be valid in most Sub-Saharan Africa, especially in Kenya and Nigeria where aflatoxin concentrations in feeds are particularly high (Table 7). A comprehensive survey on aflatoxin contamination of feeds and feed ingredients in Asia-Oceanian countries, including Malaysia, Philippines, Thailand, Indonesia, and India (Southeast Asia) showed that 30.3% of the samples contained AFB1 at an average level of 46.0 µg/kg and a maximum level of 4278.0 µg/kg [164]. Moreover, the levels of aflatoxins in commercial poultry feeds were demonstrated to be significantly higher than the maize used as ingredient in their formulation [118,165]. Nevertheless, feed contamination with aflatoxins may not necessary correlate with that of ingredients used in feed formulations, depending on the type and composition of the feed, the processing steps when applicable, and considerations of quality grading. For instance, maize for feed manufacture in Indonesia was separated into three grades of decreasing quality before being analysed for aflatoxin contents. The quality of the maize was determined visually on the basis of the proportions of foreign materials and mouldy, dead, or damaged kernels, as per the Indonesian grading system routinely practiced by the feed milling industry [166]. Unexpectedly, the results showed that aflatoxin concentrations increased from the best to the worst grade of the grains, suggesting that the grading system relying on visual inspection does not reflect *a priori* extent of contamination. The relatively high aflatoxin levels recorded in feeds of the regions (Table 7) are of concern to both animal and human health, since they are not only detrimental to livestock but can also be carried-over to human via foods derived from these animals, such as eggs, meat, and milk [167].

Table 7: Aflatoxin contamination of feeds in selected countries from Sub-Sahara African and Southeast Asian countries. Data are for total aflatoxins (B1+B2+G1+G2), unless otherwise stated in the footnotes

Country	Mixed cattle feed[a]		Sunflower seed cake feed		Maize meal		Peanut meal		Poultry feed		Various feeds[b]		References
	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	Mean (Range)	+ve (%)	
Kenya	-	-	-	-	-	-	-	-	21 (3.8-41)	100	-	-	[168]
	-	-	-	-	NS (5.13-1123)	95	-	-	-	-	-	-	[89]
	90[c] (<1-1198)	90	-	-	-	-	-	-	-	-	-	-	[169]
	-	-	-	-	-	-	-	-	-	-	52 (0-556)	78	[170]
Nigeria	-	-	-	-	-	-	-	-	-	-	115 (0-435.9	94	[170]
	-	-	-	-	176[c] (6.1-567)	47	639[c] (61-3860)	91	74[c] (0.5-760)	83	-	-	[171]
	-	-	-	-	59.7[(Gm)] (20.3-297)	100	-	-	-	-	-	-	[118]
	-	-	-	-	-	-	-	-	198 (6-1067)	76	-	-	[172]
Tanzania	-	-	6-149[d] (0-598)	57-100[d]	3.4 (2.0-16)	32	-	-	-	-	-	-	[133]
Cameroun	-	-	-	-	1.0 (≤2–42)	9.1	161 (39-950)	100	11 (<2–52)	93	-	-	[173]
South-Africa	-	-	-	-	-	-	-	-	-	-	24.9 (13-76)[e(CM)]		[174]
	14.7 (0-71.8)	52	-	-	-	-	-	-	0.7 (0-1.8)	23	-	-	[175]
Indonesia	-	-	-	-	59 (0-236)	-	-	-	-	-	-	-	[166]
Thailand	-	-	-	-	10.7 (0.9-50.3)	77	23.3 (4-106)	40	2.0 (0.5-8.5)	93	-	-	[176]
India	-	-	-	-	-	-	-	-	23.8 (0-78)	44	-	-	[165]

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Captions and abbreviations are as defined in the footnotes of Tables 7, unless otherwise specified herein; [a]Various feeds, including dairy meal, pollard, maize, maize germ, maize bran, rice germ, rice bran, wheat pollard, wheat bran, young stock, calf meal, calf pellet, sorghum, cotton seed, sunflower and pyrethrum mix, and home-made concentrates, [b]Different types of feeds analysed separately, [c]Aflatoxin B1, [d]Different regions across Tanzania and each region has a mean and incidences values; [e]Aflatoxin B1 in cottonseed meal[(CM)].

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4 Toxicity of Aflatoxins

The toxicity of aflatoxins to humans and animals through food and feed consumption and their association with acute and chronic diseases is well established [93]. However, the degree of toxicity and the toxicological effects vary greatly depending mainly on the aflatoxin type and the host. AFB1 is, by far, the most toxic aflatoxin, followed by AFG1, AFB2, and AFG2, while AFM1 has a similar toxicity as AFG1 [177-178]. The other less toxic aflatoxins and those considered to be "non-toxic" or detoxified forms are still of concern to public health due to their inherent, although weak, toxicities with potencies ranging between 0.1 and 50% compared with AFB1 [48] (see also, Table 2). Most importantly, they can invert to their highly toxic precursors in foods or after ingestion [33]. For example, aflatoxicol, which is 25 to 50% as potent as its parent AFB1, is almost entirely converted back in the liver to either the more toxic parent AFB1 or to AFM1 [1,67,179].

4.1 Major Aflatoxicosis Outbreaks

Intake of a large amount of aflatoxin in a single dose or repeatedly during a short period of time (1-3 weeks) causes an acute poisoning (hereafter designated aflatoxicosis) with typical symptoms, usually evoking severe liver damage that may lead to death [180]. Table 8 summarizes the main aflatoxicosis outbreaks that have been documented in Asia and Africa, and their circumstances. No aflatoxicosis outbreak has been reported, to our knowledge, in industrialized countries due to the low exposure which is 100-fold lower than that recorded in developing African and Asian countries (1 ng/kg bw per day vs 100 ng/kg bw per day) [178]. The first significant aflatoxicosis outbreak occurred in two Indian regions encompassing more than 200 poor setting ethnic villages with a protein deficient nutritional status who relied mainly on maize as a food source. The climate in these neighbouring Western Indian regions is typically hot desert (BWh) and hot semi-arid (BSh) characterized by low annual rainfall and chronic drought. In 1974, these regions received abundant unseasonal rain (October-November instead of the usual rainfall period of June-September), while the maize standing in the field had attained the full maturity stage to be harvested [113]. Shortly after that, an epidemic struck affecting people in family clusters and the pets sharing the same diet. The possibility of an infectious disease was ruled out, as it was not contagious and the prescription of anti-microbial drugs prove ineffective [181]. Clinical and post-mortem histopathological examinations of dead victims revealed obvious symptoms and liver lesions evoking aflatoxicosis; i.e., periportal hepatic fibrosis, and bile duct proliferation. Thin layer chromatography (TLC) analysis showed the presence of unidentified green and blue spots in extracts of necropsy liver samples and AFB1 in the serum of some patients. Moreover, the suspect maize was highly contaminated with *A. flavus* and contained aflatoxins at concentrations ranging between 6500 and 15,600 µg/kg. Exposure calculations suggested that the populations have been ingesting, through their diet, 2-6 mg of aflatoxins on a daily basis for several weeks from the start of harvest to the depletion of maize stock, which coincided with the end of the outbreak [113]. Together, these data were taken for an evidence to ascribe the epidemic to a maize-born aflatoxicosis (Table 8).

In the Sub-Sahara African region, Kenya has been the most severely afflicted by aflatoxicoses, especially in the East-central region where the prevailing climate is hot semi-arid (BSh), humid subtropical (Cwa), or oceanic tropical highland (Cwb) with frequent alternation of dry and rainy periods. Populations of these regions, mainly of the Akamba/Kamba tribe, grow maize for home consumption as the main staple food and store it by traditional means in containers that they place inside a granary or hung to the ceiling of the kitchen [91]. Two notable aflatoxicosis outbreaks were recorded in the same region of the country (Table 8). The first one occurred in 1981 after a severe shortage in rainfalls during the year 1980 followed by heavy rainy season that extended from October to May instead the normal period of October to December (short rainy season). Starting from late March to early June 1981, 20 patients, mostly from two family groups of Makueni district, were admitted to the provincial hospital with jaundice and other

symptoms suspecting a viral hepatitis [91]. Within 22 days of the early symptom onset (i.e., abdominal discomfort, anorexia, general malaise, and low-grade fever), 12 of the patients developed massive ascites and gastrointestinal haemorrhage before they died from liver failure. Among those, 6 were from the same family of 8 members including two twins who were not affected, as they were not fed the family diet. From another family of 7 members, 4 had the illness and two of them died, while the other two recovered progressively within 20 days of hospitalization. Both families were fed on inadequately stored maize as per the traditional Akamba method described above and in a wet environment [91]. In each of these families, the stored maize was found to be contaminated with AFB1 at concentrations of 12,000 and 3200 μg/kg, and AFB2 at concentrations of 1600 and 2700. As was the case in the Indian outbreak, the onset of the disease in the family members followed immediately the death of dogs sharing their diet. After necropsy, AFB1 was detected in liver samples of two deceased children at levels of 39 and 89 μg/kg, which was considered as an additional evidence supporting the causal effect of aflatoxins. Two other fatal cases tested positive for HB virus surface antigen (HBsAg), suggesting a pre-existing liver damage that increased the susceptibility to aflatoxins of the patients. Continuous dietary intake of sublethal or subclinical doses of aflatoxins in addition to protein deficiency of the diet, due to the food shortage in the previous year, were suggested to have contributed to the increased susceptibility of the victims [91].

The second episode of aflatoxicosis outbreak that occurred in Kenya in 2004 was the most significant worldwide, as it caused 317 cases with 125 deaths (Table 8). It also covered a larger zone encompassing 4 districts of more than 40,000 km^2 populated by 2.8 million inhabitants mostly of the Akamba tribe. Makueni and Kitui districts were the most severely affected (47% and 32% of cases, respectively), followed consecutively by Machakos and Thika with 6% and 4% of the total cases. In an almost identical scenario as for the former aflatoxicosis outbreaks in India (1975) and Kenya (1981), this one also occurred after unseasonal heavy rain preceded by a year of severe shortage in rain and foods, which resulted in high aflatoxin contamination of maize and increased susceptibility of nutritionally deficient rural farmers [90]. During the course of the aflatoxicosis, a survey was conducted in June 2004 in the households and market outlets to assess the aflatoxin contamination of home-grown and market maize. The highest contamination levels were recorded in samples collected from home-grown maize stored in households as compared to those of the maize sold in market outlets in the geographic area of the outbreak. In households with victims, the maize was stored under damp conditions and 48.4% of the samples contained between 20 and 8000 μg/kg of aflatoxins [182]. These considerations and the absence of viral agents, as demonstrated by serological tests in a case-control study, led the investigators to relate the aflatoxicosis to the home-grown rather than the market maize [90,182]. Yet, the contribution of market maize as a continuous source of aflatoxin intake outside the season or when the stock of the household maize is exhausted was highlighted by the authors. Overall, aflatoxin concentrations exceeded the Kenyan maximum tolerable limit of 20 μg/kg in 55% of the analysed samples collected from market maize, and the levels of contamination in samples from each of the affected districts were consistent with the number of reported cases. The highest geometric means of aflatoxin concentrations in maize, 52.91 and 35.27 μg/kg, were recorded in Makueni and Kitui, respectively. Conversely, maize samples collected from Machakos and Thika markets were the least contaminated, with geometric means of 17.84 and 7.52 μg/kg, respectively. This reflects also the aflatoxicosis ratios per 100,000 inhabitants in these districts. In Makueni and Kitui, the aflatoxicosis ratios varied from 34.8 to 77.5 in the northern areas and from 12.6 to 34.7 in the southern area, whereas they were much lower (0.66 to 12.5) in both Machakos and Thika [90]. Nonetheless, in Thika, the least affected district, the maximum aflatoxin concentration was as high as 46,400 μg/kg, which is sufficient to trigger severe aflatoxicosis after one or few servings in susceptible persons [178]. Of the total analysed samples, 7% were contaminated with more than 1000 μg/kg, whereas at the district level, samples containing such high concentrations represented 12%, 10%, 3%, and 4% of the samples collected from Makueni, Kitui, Machakos, and Thika markets, respectively [90]. In addition to the results of the survey indicating the exceptionally high contamination of the maize locally produced and consumed in these

Kenyan provinces, a case-control study was conducted separately to relate AFB1 dietary intake to the disease by titrating the biomarker AFB1-albumin adduct in serum [183]. The study, conducted on 40 selected case-patients and 80 suitable controls, demonstrated a high correlation between the titre of the adduct in the serum and aflatoxin intake via maize consumption. Moreover, the adduct titre in HBsAg negative case-patients was 22.2 times higher than that of the controls, therefore, clearly establishing the relationship between aflatoxin intake and the disease. Later, another study focused on the identification of mould species contaminating the maize responsible for the outbreak showed that out of 1,232 mould strains isolated from home-grown maize and inadequately stored at the households with victims, 97.8% and 2.1% were identified as *A. flavus* and *A. parasiticus*, respectively [184]. Isolates of *A. flavus* were largely predominated by the S-morphotype representing 71.8% of the isolates, compared with 28.2% of the L-morphotype. The study also showed that the incidence of the S-morphotype was highly correlated with the concentration of aflatoxins B in the maize, and strains of this morphotype were the exclusive contaminants of 5 samples out of 6 containing more than 1000 µg/kg of aflatoxins. Conversely, *A. parasiticus* was weakly represented (28.2% of the total isolates) and only detected in samples with aflatoxin contents lower than 260 µg/kg [184].

In late 1988, during the 9 days of the nine-emperor-gods festival held in Malaysia (http://eresources.nlb.gov.sg/infopedia/articles/SIP_1849_2011-10-21.html; accessed on 1 October, 2019), the consumption of a traditional Chinese dish called Loh See Fun was implicated in a poisoning outbreak resulting in 17 severe cases and 13 deaths. Patients were children of 2.5 to 11 years of age, with one case of a 46-years-old man, and only children died. Other patients (45 in number) who ate the same dish as the affected cases, developed similar but milder and transient symptoms; they were, hence, considered as presumptive cases and discarded from further investigation [185]. The main ingredient of the offending dish consisted of white noodle made with a of mixture of rice and corn flour. Boric acid was illicitly added to the dish by the producing factory in Kampar city (Malaysia) to extend its shelf life and enhance its sensory properties [186]. The onset of the poisoning was fairly rapid and the first symptoms evoking a Reye-like syndrome appeared within a mean time of 8.5 hours after the ingestion. The patients exhibited different symptoms, the commonly observed of which were vomiting, seizure, diarrhoea, abdominal pain, anorexia, and coma. Jaundice was generally weak at the beginning and increased in severity with time until the eventual death with liver and kidney failure. Depending on the patient, the survival time varied from 2 to 9 days with a mean of 5 days [185]. The results of clinical, analytical, and histopathological examinations ascribed the intoxication to both boric acid and aflatoxins. The boric acid poisoning (BAP) was mainly indicated by metabolic acidosis, acute renal failure, and the relatively short survival time. On the other hand, aflatoxicosis was indicated by the initial symptoms evoking Reye-like syndrome followed by liver injury and failure with bile duct proliferation, as the health status deteriorated leading to death. However, the detection of abnormally high levels of aflatoxins B1, B2, G1, M1, and M2, and aflatoxicol in various organs, including liver, kidney, heart, spleen, lung, and brain was the main supportive feature of the aflatoxicosis, although it does not exclude BAP [185]. Boric acid and aflatoxins may have acted synergistically, as indicated by diagnostic features that characterize one or the other disease but not both.

A recent aflatoxicosis outbreak was reported in the central region of Tanzania in 2016 [187]. The prevailing climate in the region is hot semi-arid (BSh) and subject to frequent alternations of drought and flood periods caused by ENSO [112]. This phenomenon induces extreme shifts in rainfall and temperature causing both severe drought and rainfall events, usually followed by increased incidence of disease outbreaks, as it creates favourable ecological conditions for microbial pathogens and their vectors to emerge. According to the latter study [112], a strong El Niño hit Tanzania in 2015-2016 and raised above normal the cases of malaria and cholera in the period of April 2015 to March 2016, which continued through 2017 for cholera. Although not mentioned in the study, this situation applies to the aflatoxicosis outbreak that occurred in the period of 14 May to 14 November 2016 (Table 8). The outbreak affected 68 individuals in family clusters and killed 20 of them. Before death, the patients presented typical symptoms of

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aflatoxicosis, i.e., jaundice, abdominal pain, vomiting, diarrhoea, and ascites. A house-to-house survey conducted in selected households including case-households with victims and those without (controls), showed that more than 50% of the cases were children below 15 years-old who had eaten home-grown maize contaminated with both aflatoxins and fumonisins at abnormally high levels [187]. Aflatoxin contamination in samples collected from case-households was significantly higher than those of controls (10-51,100 μg/kg versus 2.4-285 μg/kg). Fumonisins were detected in the maize sampled from case-households at concentrations ranging from 945 to 12,630 μg/kg. Of the maize samples contaminated with both mycotoxins, 80% exceeded the regulatory standards of 10 μg/kg and 2000 μg/kg for total aflatoxins and fumonisins, respectively. Moreover, the titres of aflatoxin-albumin adduct in the serum of case-patients usually exceeded 1000 pg/mg and were 3.6 to 8.2 times higher than in the serum of controls (36-32,800 pg/mg vs 10-4020 pg/mg) [187]. The increase in aflatoxin-albumin adduct titre is a strong indication of the causal link between aflatoxins and the outbreak, the severity of which may have been increased by an additive effect of fumonisins [188].

According to the magazine "outbreak news today", during the period of June 20 to July 13, 2017, two clusters of 8 children from two different villages of Kiteto District (Manyara region), North Tanzania, were admitted to the hospital for suspicion of aflatoxicosis [189]. They were presenting the common symptoms of acute aflatoxicosis, namely general malaise, loss of appetite, vomiting, abdominal distension and pain, dark stools without diarrhoea, and jaundice. Three cases of the first cluster, consisting of five children (three to nine years-old), died shortly after the admission. The 3-years-old child died after two days and the other four were transferred to the regional referral hospital of Dodoma for more intensive care; and two of them died two days later. On July 13, 2017, the three children (one of four-years-old, and two of ten years-old) of the second family cluster were admitted to the hospital with similar symptoms as the previous patients plus an altered mental status [189]. The four-years-old child died within hours after admission, meanwhile the other patients were referred to the regional hospital with the two survivors of the first cluster. As per the date of the report (July 24, 2017) the four survivors were still hospitalized and there was no update on the situation to our knowledge. All the children were reported to have consumed improperly stored maize, which in conjunction with the symptoms suggests that the disease is likely to be an aflatoxicosis [190].

In addition to the above-mentioned aflatoxicosis outbreaks, some sporadic cases have contributed to increase the scientific knowledge on the toxicity of aflatoxins. For example, the death of a Ugandan teenager in 1967 who had been fed regularly on mouldy cassava as a staple meal and the association of his death with aflatoxicosis, as evidenced by the typical liver lesions observed upon post-mortem histopathological examination and the high contamination of the cassava meal (1700 μg/kg), was the first demonstration of the acute toxicity of aflatoxins in humans [191]. Also, intentional ingestion by a 25-years-old laboratory female worker who attempted to commit suicide of 5.5 mg of pure AFB1 over two days and another dose of 35 mg, six months later, over two weeks and developed only minor transient symptoms (rash, nausea, and headache) gave some insights on the difference in susceptibility to aflatoxins among individuals [192].

604 **Table 8**: Significant aflatoxicosis outbreaks in Asia and Africa

Country	Region	Year (Period)	Number of cases	Number of deaths (% of fatality rate)	Associated Food	Level of contamination (mg/kg)	Specific remark	Reference
India	Western (Rajasthan-Gujarat)	1974 (October-November)	397	106 (26.7)	Maize	6.3-15.6	Heavy unseasonal rain after drought and faulty storage conditions	[113]
Kenya	East-Central (Makueni)	1981 (March-June)	20	12 (60)	Maize	3.2 and 12.0	Rain shortage in the year preceding the outbreak followed by prolonged high rainy season and faulty storage conditions	[91]
	East-Central (Makueni-Kitui-Machakos-Thika)	2004 (January-July)[a]	317	125 (39.4)	Maize	1.0-46.4	First study relating aflatoxin-albumin adduct to human aflatoxicosis and its use as a biomarker	[90,182]
Malaysia	Perak state	1988	17[b]	13[c] (76.5)	Chinese[d] noodles	NS	Possible additive effect of boric acid and aflatoxin	[185-186]
Tanzania	Central	2016 (May-November)	67	20 (30)	Maize	10-51,100	Possible additive effect of fumonisins High titres of aflatoxin-albumin adduct in the serum of patients used as evidence for the aflatoxicosis	[187]
	Northeast	2017 (June-July)	8	4 (50)	Maize	NA	Evidence based on symptoms and consumption of maize reported to have been inadequately stored	[189]

605 [a]A peak was reached in May to Mid-July; [b]16 children 2.5-11 years of age a 49-years old adult; [c]All children, and they died within hours of
606 the intoxication and the percentage of death excludes 45 cases not admitted to the hospital because they developed only mild symptoms;
607 [4]Dish called "Loh See Fun" suspected to have been preserved with banned boric acid. *Abbreviations*: BAP: Boric acid poisoning; the others
608 as in the Tables above

4.2 *Chronic Diseases*

Repeated exposure to low doses of aflatoxins over a lifetime causes chronic diseases, the most frequent and severe of which is cancer. Although dietary intake of aflatoxins has been classically associated with primary liver cancer, i.e., HCC and bile duct hyperplasia [193], other organs such as the kidney, the pancreas, the bladder, bone, viscera, etc. have also been reported to develop cancer upon exposure to these mycotoxins [194]. In addition, aflatoxins were reported to cause lung [195] and skin [196] occupational cancers via inhalation and direct contact, respectively. In fact, chronic exposure to aflatoxins causes a range of other severe diseases, including immunosuppression, teratogenicity, mutagenicity, cytotoxicity, and estrogenic effects in mammalians [197]. Moreover, aflatoxins are believed to be involved in nutritional disorders, such as kwashiorkor and growth faltering probably by interfering with the absorption of micronutrients (e.g., zinc, iron, and vitamins), proteins synthesis, and metabolic enzymes activities [180,198]. In domestic animals, feeds contaminated with sub-lethal doses of aflatoxins induces impaired productivity and reproduction, increased susceptibility to diseases, and reduced quality of the foods they produce [178]. Despite the insidious character of chronic aflatoxin-induced diseases, their impact on public health globally is more severe and more costly than acute aflatoxicosis. Although, the latter induces hundreds of deaths at once in an intermittent manner, it can be prevented or interrupted upon analysis of suspect crops/foods, e.g., evident mould growth, and their disposal if aflatoxin levels are found to be too high.

Liver cancer is one of the most common and deadly type of cancer diseases whose occurrence has been strongly correlated to dietary exposure to aflatoxins, which is enhanced in the presence of other risk factors [180]. Notably, chronic infections with HB was shown to increase by up to 60 times the potency AFB1 [199]. According to the most recent statistics given by the global cancer observatory of the IARC (http://gco.iarc.fr, accessed on September 1st, 2019), 841,080 new cases of liver cancer causing 781,631 deaths were recorded globally in 2018. This corresponds to an age-standardized incidence rate of 9.3 per 100,000 and mortality rate of 93% ranking as the fifth cancer type and the first cause of cancer-induced mortality. Africa and Asia continue to be the leading continents in terms of new cases recorded each year, with 64,779 (7.7%) and 609,596 (72%) cases respectively, together representing about 80% of the total cases in the world. Aflatoxin B1 alone was estimated to cause 25,200 to 155,000 cases each year [7,200], 40% of which occur in the sub-Saharan Africa only [180] where aflatoxin-induced liver cancer accounts for a-third of all liver cancer cases registered in the whole African continent [201]. At the country level, China has the highest incidence of liver cancer in the world, with the vast majority being recorded in the Southern part of the country where the two main synergistic causative agents, exposure to dietary aflatoxins and HB chronic infections, are endemic and highest [193].

5 Mechanisms of Toxicity

Aflatoxins exert various toxicological effects with different mechanisms, most of which are not fully elucidated yet. Intensive research has been carried out to investigate the mechanisms of the toxicity of aflatoxins to provide a scientific basis for the design of preventive and control means, as well as for regulatory purposes. The mutagenic effects of AFB1 have been the focus of most studies since their discovery and were ascribed mainly to their intermediate metabolite AFB1-exo-8,9 epoxide (AFBO) [1]. As a highly unstable molecule, AFBO reacts with cellular macromolecules, including nucleic acids, proteins, and phospholipids to induce various genetic, metabolic, signalling, and cell structure disruptions [202-204]. However, increased evidence is being built up demonstrating equally dramatic or higher effects of AFB1 on cell function and integrity through the induction of oxidative stress (OS) [205-207]. Figure 2 summarizes the different toxicity mechanisms of AFB1 involving AFBO and OS to cause genotoxicity, immunotoxicity and acute intoxication by acting on genomic DNA.

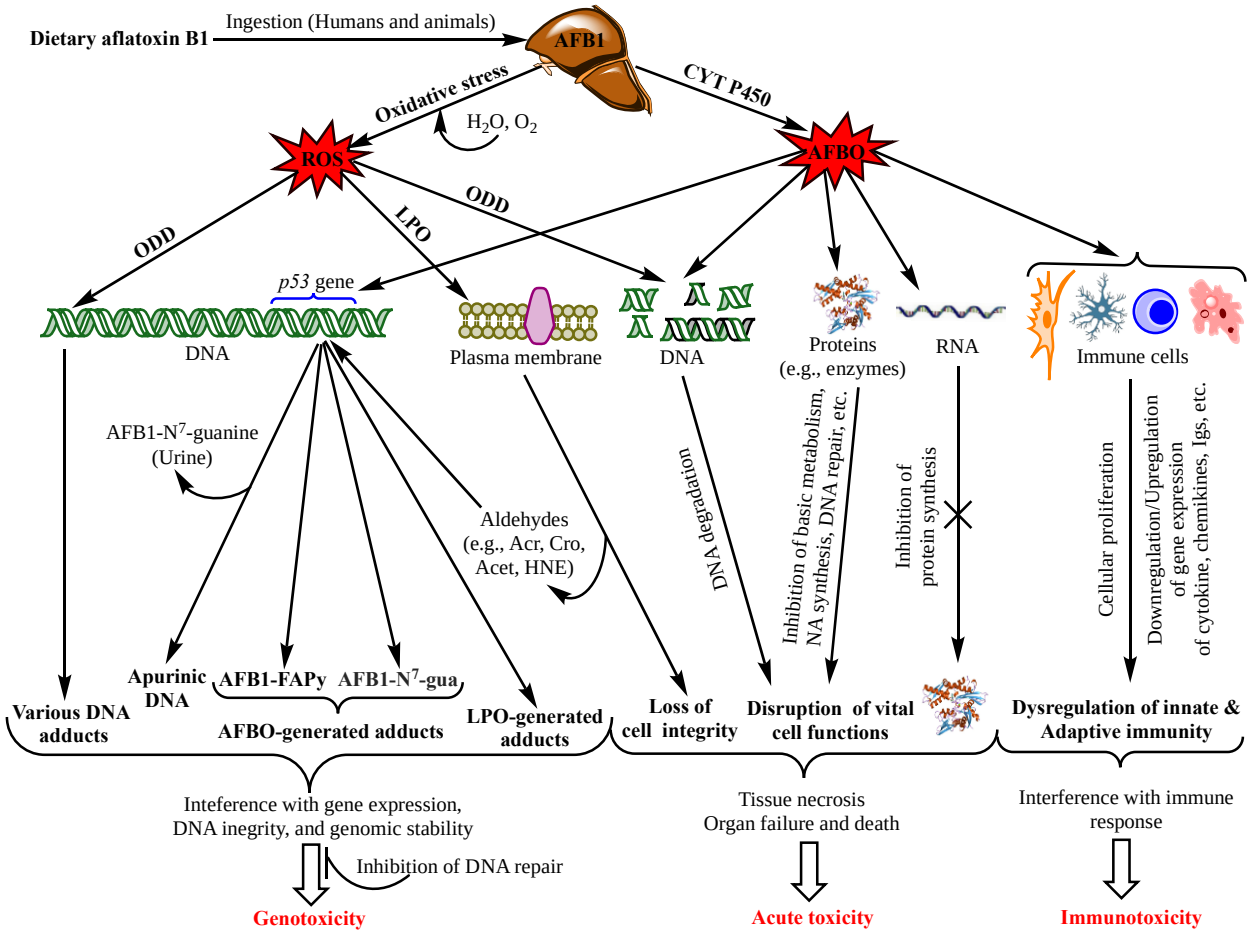

Figure 2: Main aflatoxin B1 toxicity mechanisms mediated by the oxidative stress and AFBO (see text for explanations). NB: ROS also affect proteins, RNA molecules, and immunity as does AFBO (not shown in the figure. For details, see [208]). *Abbreviations*: AFBO: Aflatoxin B1-exo-8,9-epoxide; NA: Nucleic Acids; ROS: Reactive Oxygen Species; LPO: Lipid Peroxidation; ODD: Oxidative DNA Damage; Acr: Acrolein; Cro: Crotonaldehyde; Acet: Acetaldehyde; HNE: 4-Hydroxy-2-Nonenal; uFA: Unsaturated Fatty Acids; IL1β: Interleukin 1β, IL6: Interleukin 6; TNFα: Tumour Necrotizing Factor α; P-dG: Cyclic Propano-Deoxyguanosine; Igs: Immunoglobulins. See text for the other abbreviations.

5.1 Genotoxicity and cancer diseases

5.1.1 AFBO-Mediated Genotoxicity

AFBO has long been considered as the ultimate metabolite responsible for the genotoxic effects of AFB1 as well as other aflatoxins bearing a double bond between carbons C8 and C9 in the furan ring [197,209]. The mechanisms of toxicity mediated by this AFB1 reactive metabolite are the best understood and have been extensively reviewed [205,210-212]. Upon ingestion, AFB1 is absorbed in the duodenum and reaches the liver where it is bioactivated by action of various microsomal cytochrome enzymes (CYT P450). These are monooxygenases that catalyse the oxidation of the C^8=C^9 double bond in the furan ring yielding AFB1-exo and -endo 8,9 epoxide stereoisomers, with the former isomer being >1000 times more reactive/toxic than the latter [213]. Different CYP450 isozymes are responsible for the bioactivation of AFB1 depending on the host, the organ, and the sub-cellular component. In humans, among 57 CYP450 identified

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isoenzymes, the microsomal CYP1A2, 3A4, 3A5, 3A7, 2A3, and 2B7, the hepatocytic 3A3, and the lung CYP2A13 are the principal isozymes responsible for AFB1 bioactivation in the respective organs [214-215]. In the liver, the bioactivation is essentially catalysed by CYP1A2 or 3A4, with predominating action under low or high exposure conditions, respectively; CYP1A2 predominates under the actual food contamination levels and is also responsible for the transformation of AFB1 into the less toxic endo-epoxide isomer [216-217]. In animals and insects, various CYP450 isozymes, including CYP1A1, 1A, 1A2, 2A5, 2A6, 3A, 3A4, 3A13, and 321A1, were reported to catalyse the bioactivation step depending on the species and the organ where they are produced. The specific roles of CYP450 isozymes in AFB1 metabolism and their distribution in different hosts and organs were reviewed elsewhere [218].

Once released, AFBO intercalates the DNA and binds covalently, upon alkylation reaction, to the N^7 atom of guanine residue forming a stereospecific aflatoxin-DNA adduct, *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB1 (AFB1-N^7-gua) [211], most frequently (60-80%) at the third guanine residue of the codon 249 ($^{5'}$-AG*G-$^{3'}$) on *p53/PT53* tumour suppressor gene [219-220]. While bound to the DNA molecule, AFB1-N^7-gua adduct is highly unstable, due to its positive charge, and releases itself leaving an apurinic DNA molecule (AP). However, the imidazole ring may be opened under slightly alkaline conditions to form two stable isomers *cis-* and *trans*-AFB1-formamidopyrimidine (AFB1-FAPy) adducts, also called minor and major AFB1-FAPy adducts, respectively (Figures 2 and 3). These three AFBO-induced DNA lesions (AP, AFB1-N^7-gua, and AFB1-FAPy) have been known as the main precursors of AFB1 genotoxic and carcinogenic effects (Figure 2). Among them, AFB1-FAPy was reported to be the most mutagenic due to its persistent DNA damage [221-222], which was ascribed to the less helix-distorting lesions it induces compared with those caused by AFB1-N^7-gua, thereby hindering DNA repair [205,210]. AFB1-FAPy lesions are essentially repaired by the nucleotide excision repair (NER) mechanism, which is contingent to the extent of DNA helix distortion for the recognition of damaged sites; the more distorted the site, the easier it is to be recognized by the repair proteins [205,223-224]. Yet, the higher mutagenicity of AFB1-FAPy lesions may not be explained solely by its refractory behaviour to NER repair, as they can also be repaired by the less helix-distortion sensitive mechanism of base excision repair (BER) [225]. BER involves a site-specific recognition DNA lesions by glycosylases followed by excision of the damaged sites and replacement by the correct base [226]. However, it is now well established that exposure to aflatoxins induces various epigenetic changes in repair genes that impedes BER. For example, hypermethylation of the promoter of *NEIL1* (Nei Like 1) gene coding for a DNA glycosylase (NEIL1) that plays a key role in BER was recently shown to reduce the excision efficiency in AFB1-FAPy adducts by transcriptional repression of the gene [227]. The repair of AFB1-FAPy lesions may be further restricted in humans due to the widespread polymorphic variants producing catalytically inactive NEIL1 enzyme [228]. Polymorphism in other human DNA repair genes, such as *XPC, XPD, XRCC1, XRCC3, XRCC4, XPD,* and *XRCC7*, has been reported as an additional factor that increases the risk for aflatoxin-induced HCC, particularly in high exposure environments [226,229-232]. This risk is exacerbated with simultaneous polymorphism of repair genes and phase II-enzyme detoxifying genes, as was demonstrated for the combined polymorphisms of *XRCC1* (involved in BER repair) with *GSTM1* and *HYL1*2* (coding for GST and microsomal epoxide hydrolase, respectively) [231-232]. Meanwhile, the effect of AFB1-detoxifying gene polymorphism alone on the increase of HCC risk remains controversial [231-237]. It should be pointed out, however, that most of the reports on the interaction of polymorphisms with AFB1 exposure to increase HCC risk were case-control studies conducted on highly exposed populations of Guangxi (China) and The Gambia [226,237]. The rationality of these studies suffered biased uncertainties due to limited access to HCC-case patients and the possible interference with other factors, such as smoking, drinking, and impaired liver functionality of HCC cases yielding imprecise biomarker estimates. Nevertheless, there is a consensus that interaction between exposure to AFB1 and polymorphism of the repair genes to increase HCC risk is likely, especially in high-risk environments, e.g., high exposure, chronic hepatitis virus infections. Moreover, higher resistance to DNA repair of AFB1-FAPy adduct was attributed to its ability to stabilize the double helix

owing to the way of its insertion between the helices [223]. Once intercalated between the DNA helices at the G:C site, FAPy stacks with neighbouring base pairs to stabilize the double helix, which is enhanced in the presence of the formamido group that probably establishes intra-strand sequence-specific hydrogen bonding within the helix [238]. Nevertheless, irrespective of the lack of a clear mechanistic explanation, various observations and mutational studies on the stability of lesions and repair efficiencies have established the implication of AFB1-FAPy adduct in the vast majority of AFB1-induced mutations and, therefore, its higher genotoxicity compared with the other AFBO-induced lesions [205,210-211,239].

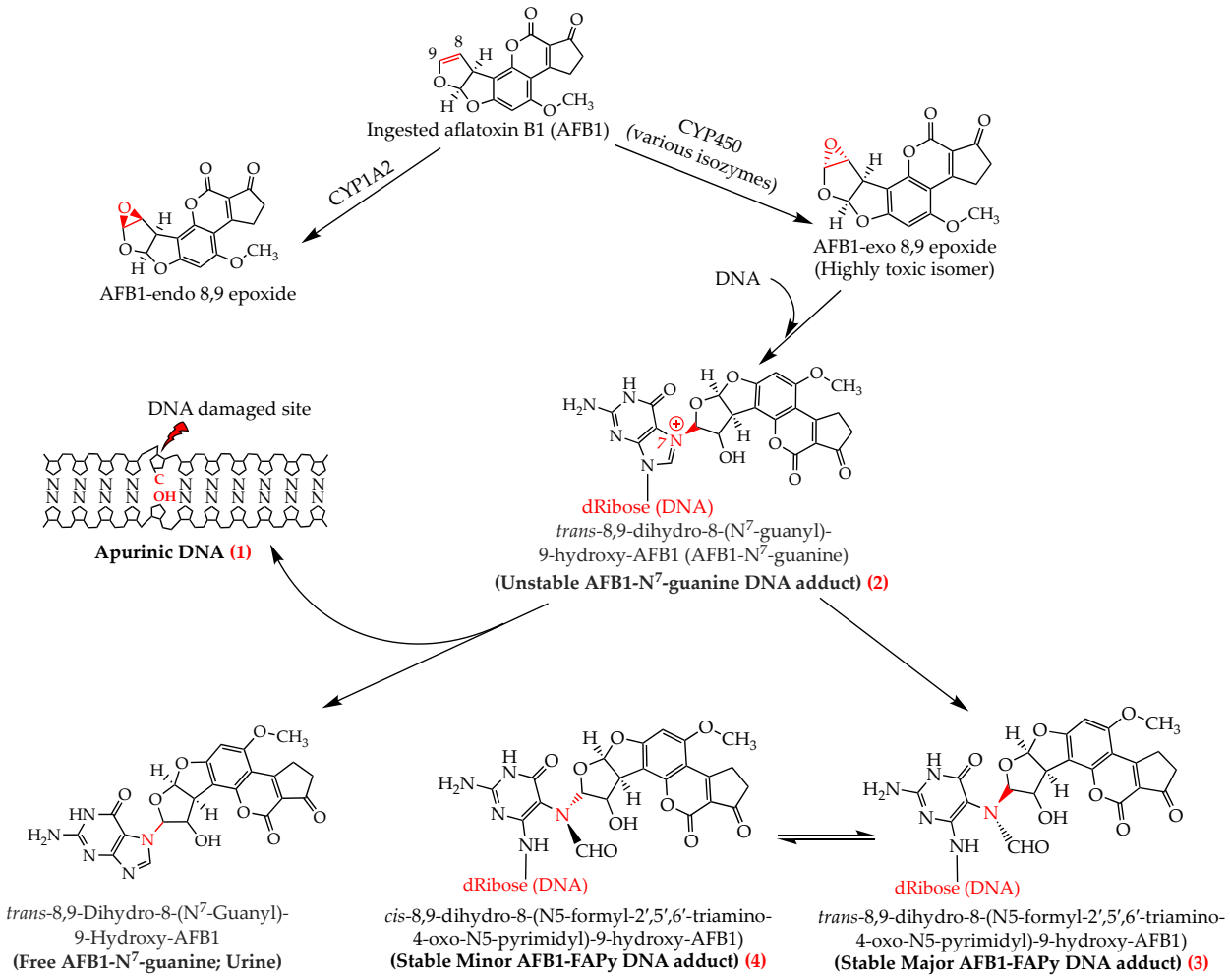

Figure 3: Activation of aflatoxin B1 and its interaction with the DNA leading to the formation of aflatoxin DNA adducts causing three main DNA lesions, AFB1-N^7-guanine (1), apurinic DNA (2), and AFB1-FAPy (3 and 4), involved in mutagenicity and carcinogenicity. Upon furan ring opening to stabilize the AFB1-N^7-gua DNA adduct, the "*cis*" (minor) rotamer of AFB1-FAPy is formed first and is then transformed into the "*trans*" (major) rotamer to an equilibrium where the major rotamer is predominating (2:1; major to minor) [240].

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Failure to repair the DNA damaged by any of the above-mentioned lesions leads to transversion mutations, predominantly G→T (80%), of the third base $^{5'}$G of the codon 249 on *p53* gene; in few instances, the second base of the codon or G→A transversions have been reported [210,220]. As a result, the mutant expresses a non-functional protein where the serine residue at the position 249 is substituted for arginine. The resulting altered protein, pR249S, cannot bind to DNA molecules and hence loses its transactivation capacity towards a multitude of *p53*-dependent gene promoters responsible for various vital cellular functions, including cell cycle arrest, senescence, and apoptosis, eventually leading to tumorigenicity [241-242]. Two of the most known *p53*-dependent regulatory genes involved in cell cycle progression and/or apoptosis signalling pathways are *CDKN1A* (cyclin-dependent kinase inhibitor 1A) and *PUMA* (p-53 upregulated modulatory apoptosis). In normal functioning conditions, exposure to genotoxic insults upregulates the latter genes by the transcriptional factor p53 to express the effector proteins CDKN1A/p21 and PUMA, respectively (Figure 4).

p21, also known as $p21^{WAF1/Cip1}$, regulates negatively the progress of cell division mainly through the inhibition of cyclin-dependent kinases (CDKs) or proliferating cell nuclear antigen (PCNA) as illustrated in Figure 4. Various mechanisms have been proposed for p53-driven cell cycle arrest emphasizing the role of CDKN1A gene product (p21 protein) which antagonizes with CDKs responsible for the initiation of a cascade of events leading to the repression of many genes involved in the progress of the cell cycle at different checkpoints [242-245]. A more recent mechanism suggests an indirect repression of cell cycle progression via p21-DREAM-E2F/CHR (p53-DREAM) pathway, wherein a transcriptional repressor, DREAM (dimerization partner, RB-like, E2F and multivulval class B), binds to E2F and CHR (cell cycle gene homology region) promoter sites and downregulates the transcription of more than 250 genes controlling the progression of the whole cell cycle at different stages from G0 to cytokinesis [246-247]. DREAM is a multi-subunit complex composed of a core multivulval class B (MuvB) complex associated with E2F4 or E2F5, dimerization partner (DP), and proteins p107 or p130 (also called RB-like proteins 1 and 2, respectively) (Figure 4A). However, for p107 and p130 to bind and activate the other subunits of the DREAM complex, they should be in their hypo-phosphorylated states, which requires active p21 to inhibit cyclin-CDK complexes, e.g., cyclinE-CDK2 and cyclin D-CDK4/6, responsible for their hyper-phosphorylation [246]. Sequestration of PCNA by p21 can also evoke cell cycle arrest at a given stage of the cycle by blocking DNA replication and repair requiring PCNA as a co-factor for the activity of DNA polymerase δ [243] (Figure 4B). Although these studies have been carried out on different cell types and organs, and with different DNA-damaging or -nondamaging stimuli, the results can apply to AFB1-induced DNA damage. An intraperitoneal administration of AFB1 to mice at a daily dose of 20 µg/kg bw for up to 21 days induced overexpression of p21 with concomitant downregulation of cyclin D1 and CDK4, which inhibited the formation of cyclin-CDK complexes, ultimately leading to cell cycle arrest and apoptosis [248]. However, the study demonstrated that although both cell cycle arrest and apoptosis were *p53*-dependent, the upregulation of p21 expression was not involved in apoptosis. In fact, it is well established that p21 plays an antagonistic dual role, not yet well understood, as it can either restrict or promote apoptosis depending on many factors, such as the extent of DNA damage, the type of stimulus, the tissue, the type of cell line, the subcellular localisation, chemotherapy treatment (if any), etc. [244-245]. In the cytoplasm, p21 primarily exerts an anti-apoptotic, i.e., tumour promoting, action whereby it inhibits key enzymes responsible for the induction of apoptosis or the transcription factors responsible for the transactivation of genes coding for pro-apoptotic proteins (Figure 4C). The anti-apoptotic effect while the cell cycle is arrested needs further clarifications for the circumstances of its occurrence and regulatory mechanisms that trigger the switch from pro-apoptotic to anti-apoptotic action and vice-versa. Presently, the prevailing explanation considers that, in conjunction with the cell cycle arrest, p21 inhibits apoptosis to ensure cell survival during the pause of cell cycle arrest in order to provide an opportunity for DNA repair before proceeding with the normal growth cycle. However, in case of a severe damage and impaired DNA

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repair, the role of p21 located in the nucleus is switched to be pro-apoptotic and activates the caspase cascade driving cell death [243-244,249].

Cells exposed to various genotoxic and nongenotoxic stimuli may undergo *p53* dependent or independent apoptosis; however, genotoxic stimuli, e.g., exposure to AFB1, causing severe DNA damage trigger essentially *p53*-dependent apoptosis involving two main regulatory proteins of the family of the Bcl-2 homology domain 3 (BH3)-only, PUMA and NOXA, with PUMA being involved in virtually all *p53*-dependent apoptotic activities [250]. PUMA is transcriptionally upregulated by p53 protein to antagonize with pro-survival proteins of the Bcl-2 family that inhibit constitutively the pro-apoptotic pore-forming BAX (Bcl-2-associated X protein) and/or BAK (Bcl-2 antagonist/killer) proteins. The inhibition of the pro-survival proteins activates BAX/BAK which oligomerize to form pores in the outer membrane of mitochondria allowing leakage of pro-apoptogenic proteins that initiate a cascade of events ending with the activation caspases directly involved in cell death (Figure 4D).

Under DNA damaging conditions in *p53* mutants, *CDKN1A* and *PUMA* genes remain repressed due to the lack of functional p53 transcriptional factor. Consequently, CDKs, PCNA, and pro-survival Bcl-2 proteins are relieved leading to uninterrupted cell cycle with a poor repair machinery and overexpression of *CDK* genes, among multiple other physiological dysfunctions (Figure 4). While the restriction of cell cycle arrest increases the likelihood for unfaithful DNA replication and accumulation of mutations among other metabolic and signalling dysfunctions, the overexpression of CDKs was shown to play crucial role in tumour development and promotion [251-252]. Restriction of apoptosis, a major physiological function for the elimination of senescent, damaged, or stressed cells, due to the repression of PUMA and cytoplasmic p21 in *p53* mutants, exacerbates the risk for cancer diseases. It is also well established that any disruption in the expression and the signalling pathways involving pro-apoptotic or pro-survival proteins of the Bcl-2 family members not only promotes cancer but also increases its resistance to chemotherapy [253]. In fact, in addition to the restriction of cell cycle arrest and apoptosis, *p53* mutations de-regulate the genetic expression of a plethora of genes controlling various other cellular functions and metabolic pathways, such as the oxidative phosphorylation, glycolysis, stemness, signalling, DNA repair, maintenance of genomic stability, etc. part or all of which are involved in tumour suppression, as has been extensively reviewed recently [212,241-242,247,254-256]. This also explains why *p53* mutations are associated with more than 50% of human malignancies, including aflatoxin-induced HCC [219].

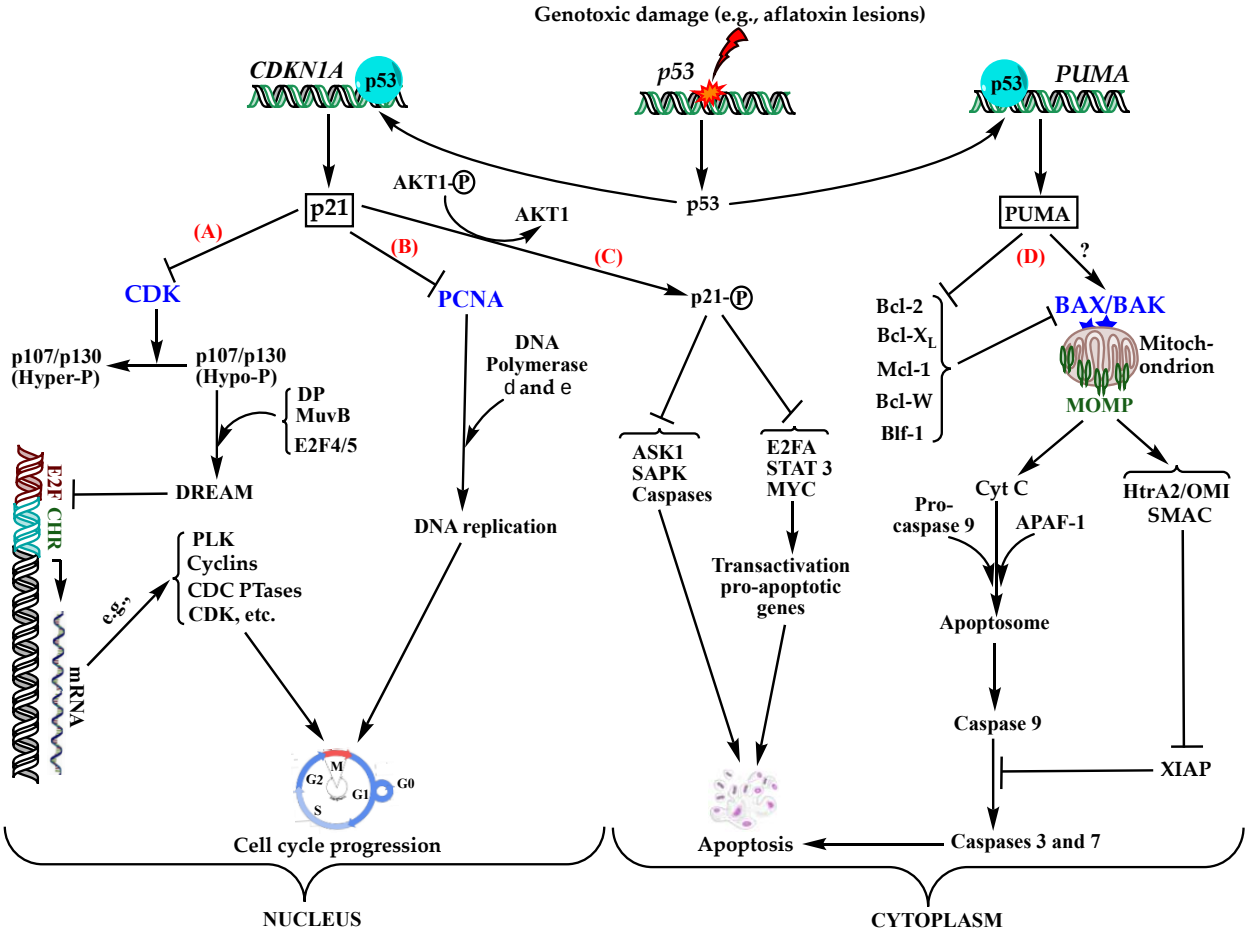

Figure 4: Main mechanisms used in normally functioning cells to induce cell cycle arrest or apoptosis as a response to DNA damage affecting *p53* gene to inhibit cell cycle progression in the nucleus (A) and (B), or apoptosis in the cytoplasm (C) and (D). **(A)** p21, as a potent inhibitor of CDKs, inhibits the phosphorylation of p107 and p130 proteins, which in their hypo-phosphorylated states can bind to MuvB core complex, E2F4-5, and DP and form an active DREAM complex. Once formed, DREAM binds to E2F and CHR promoters and represses the transcription of many genes, e.g., polo-like kinases (PLK1), cyclins A, B1, and B2, CDK1, CDCs 20, 25A, and 25C, MCM5, BIRC5, etc., involved in the progress of the cell cycle at different stages and checkpoints, thereby arresting the cell cycle at any stage of the progression depending on the gene(s) inhibited [246]. In the absence of p21, CDKs remain active and hyper-phosphorylate p107 and p130 preventing them from binding to the other DREAM components, thereby leaving E2F and CHR promoter sites free to bind transcriptional activators that, on the contrary, promote the cell cycle progression [242,247]. **(B)** p21 interacts with PCNA in the nucleus and prevents it from binding to δ subunit of DNA-polymerase, which blocks DNA replication as well as DNA repair among other functions ensuring the fidelity of DNA duplication [249]. **(C)** p21 can be phosphorylated by the serine threonine kinase AKT1 and prevented from translocating into the nucleus; in the cytoplasm, it acts as an anti-apoptotic factor that inhibits pro-apoptotic enzymes, such as ASK, SAPK, and different caspases. It also inhibits transcriptional factors, such as E2F1, STAT3, and MYC preventing the transactivation of pro-apoptotic genes [242-244]. **(D)** p53 transactivates *PUMA* gene as the major p-53-dependent mechanism for intrinsic apoptosis induction. Under normal conditions and in the absence of stimuli, apoptosis is restricted by 5 pro-survival proteins of the Bcl-2 family; Bcl-2, Bcl-X_L, Mcl-1, Bcl-W, and Blf-1. Upon exposure to genotoxic stimuli, such as aflatoxins, p53 upregulates the expression of PUMA, a member of the Bcl-2 homology 3 (BH3)-only family, which inhibits all of the five pro-survival Bcl-2

proteins, thereby de-represses the pro-apoptotic proteins BAX and/or BAK. This initiates mitochondrial damage allowing leakage of pro-pro-apoptotic proteins through MOMP formation upon oligomerization of BAX/BAK, namely cytochrome C, HtrA2/OMI, and SMAC, which cooperatively induce apoptosis; cytochrome C binds APAF-1 and procaspase 9 to form an apoptosome and activate caspase 9 triggering the caspase cascade directly involved in apoptosis. Yet, caspase cascade can still be blocked by the pro-survival protein XIAP inhibitory to caspases 9 and 3. To proceed with apoptosis, SMAC and HtrA2/OMI combine to inhibit XIAP and relieve the caspases [257]. ?: In the absence or saturation of pro-survival Bcl-2 proteins, PUMA can directly activate BAX/BAK to resume the apoptosis process starting from MOMP formation, but this needs further studies to be ascertained [258]. *Abbreviations:* Bcl-2: B cell lymphoma-2; BH3-only: Bcl-2 homologue 3-only; Bcl-X_L: B cell lymphoma extra-large; MuvB: Multivulval class B; DP: Dimerization partner; DREAM: Dimerization partner, RB-like, E2F and multivulval class B; CHR: Cell cycle gene homology region; PLK: Polo-like kinase; CDK: Cyclin dependent kinase; CDC: Cell division cycle; MCM: Minichromosome maintenance; BIRC: Baculoviral inhibitor of apoptosis repeat-containing 5; PCNA: Proliferating cell nuclear antigen; ASK: Apoptosis signal-regulating kinase; SAPK: Stress-activated protein kinase; STAT3: Signal transducer and activator of transcription; MYC: Myelocytomatosis; PUMA: p53-upregulated modulatory apoptosis; Mcl-1: Myeloid cell leukaemia-1; Blf: BCL-2-related protein isolated from foetal liver; BAX: Bcl-2-associated X protein; BAK: Bcl-2 antagonist/killer; MOMP: Mitochondrial outer membrane permeabilization; Cyt C: Cytochrome C; APAF-1: Apoptotic protease-activating factor 1; SMAC: Second mitochondria-derived activator of caspases; XIAP: X-linked inhibitor of apoptosis protein; HtrA2/OMI: High-temperature requirement protein A2; Hypo-P: Hypo-phosphorylated; Hyper-P: Hyper-phosphorylated.

5.1.2 Oxidative Stress-Mediated Genotoxicity

Although the mutagenicity of aflatoxins has been primarily attributed to the formation of aflatoxin-N^7-gua DNA adducts discussed above, it is being increasingly evident that it also arises from the oxidative stress (OS) produced by AFB1 metabolism. The OS acts directly on the DNA to induce the so-called oxidative DNA damage (ODD) or indirectly via the formation of by-products from lipid peroxidation (LPO) of membrane phospholipids [206,259-260] (Figure 2). Processing of AFB1 in the liver by CYP 450 enzymes induces OS releasing excessive amounts of reactive oxygen species (ROS) that can attack nitrogen bases and deoxyribose moieties of the DNA and generate more than 100 different DNA adducts [206,261-262] (Figure 2). The most known and best studied of these adducts is the 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-hydroxydeoxyguanosine, 8-oxo-dG, 8-OH-dG) derived from the oxidation of the DNA guanine residue by the hydroxyl radical generated by the OS, which is commonly used as a biomarker for oxidative DNA damage [206,261,263]. Intraperitoneal injection of AFB1 to rats increased, in a dose- and time-dependent manner, the levels of 8-oxo-dG in the liver, which was prevented by a pre-treatment of rats with the antioxidants selenium and deferoxamine, thereby confirming the relationship between the adduct and the oxidative stress induced by the aflatoxin [264]. Likewise, intraperitoneal injection of a single tumorigenic dose of 50 mg/kg AFB1 to mice increased by about three-fold the levels of 8-oxo-dG in alveolar macrophages and non-ciliated bronchiolar cells (Clara or Club cells) preparations isolated from mice scarified 2 h after the treatment; no such increase, however, was observed in liver tissues of the mice [265]. Consistent with these findings regarding the absence of the adduct in the liver, a recent study showed no significant increase in seven ROS-modified bases, including 8-oxo-dG, in the liver tissues of rats treated with 7.5 mg/kg AFB1, as compared with control rats (untreated); whereas the levels of 8,5′-cyclo-2′-deoxyadenosine, another DNA adduct from oxidative attack of the adenine base, increased significantly compared with background levels in control rats [220]. The extent of oxidative DNA damage, the type of adduct produced, and the efficiency and speed in DNA repair were reported to be dependent on the species, organ, tissue, sub-cellular component, and cell cycle [206]. The lung appears to be the most common target for DNA damage with 8-oxo-dG accumulating mainly in mitochondria and the nucleus [263,266-267]. A recent study demonstrated that AFG1 upregulated the expression of tumour necrosis

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factor (TNF)-α and CYP2A13 in mice alveolar type II (AT-II) cells of lung tissues and in vitro in human AT-II-like cells (A549), which mediate an inflammation with increased numbers of γ-H2AX- and 8-OHdG-positive cells in the inflamed tissues [268]. According to the authors, the inflammatory reaction induced by TNF-α upregulates the expression of CYP2A13, which in turn sustains active metabolism of AFG1 leading to ODD, as evidenced by the increased expression of the DNA damage marker γ-H2AX. Like AFBO-derived DNA adducts, 8-oxo-dG lesions mediate G→T transversion mutations but they do not specifically target the *p53* gene and they involve different mechanism and DNA polymerases [264].

Regardless of the source of OS-induction, this is a frequent phenomenon in cells, which is normally counteracted by physiological mechanisms involving antioxidant systems consisting of enzymatic antioxidants, such as superoxide dismutase, catalase, and glutathione peroxidases, and thioredoxin, or antioxidants metabolites [206,269]. It also triggers modulatory signalling pathways to balance the associated inflammatory reactions, among which Nrf2 (Nuclear erythroid-2 related factor-2)/ARE pathway was reported to play a pivotal role. In this pathway, the Nrf2, a transcription factor that is normally sequestered by Keap 1 (Kelch-like ECH-associated protein) is liberated and upregulates ARE gene expression of detoxification enzymes, such as haemoxygenease-1 (HO-1), which inhibits pro-inflammatory cytokines and activates anti-inflammatory cytokines [270]. Yet, the above-mentioned OS-modulatory means may be of limited efficacy to prevent DNA damage that should be repaired before replication in order to preserve the genomic stability and prevent cumulative mutations and genotoxicity [206]. The 8-oxo-dG lesions are primarily repaired by BER mechanism using, for example, 8-oxoguanine DNA glycosylase 1 (OGG1) enzyme, a multifunctional DNA glycosylase that specifically recognizes the damaged base and excises it by breaking the N-glycosidic bond. The enzyme then cleaves the DNA backbone leaving an AP site to be filled by the appropriate nucleotide in subsequent steps using specialized DNA polymerases [260]. However, when the amounts of ROS are too high to be balanced by cellular antioxidant defence mechanisms, the DNA damages cannot be repaired timely and they accumulate to produce various genetic abnormalities, including erroneous gene expression, multiple mutations, genomic instability, and eventually tumorigenicity [206]. The production of 8-oxo-dG by OS has indeed been reported to be an important means by which AFB1 causes cancer in various organs in humans and animals [220,264,271].

As was mentioned above, AFB1 can also induce DNA damage by OS indirectly via the production of ROS which, in turn, attack oxidatively membrane phospholipids and release different mutagenic aldehydes (Figure 2). Among 33 known LPO-derived aldehydes, malondialdehyde (MDA), acrolein (Acr), 4-hydroxy-2-nonenal (HNE), and 4-oxo-2(E)-nonenal (ONE) are the most predominant and can react with DNA bases to generate pro-mutagenic exocyclic DNA adducts leading to genomic instability and possibly to carcinogenicity [272-278]. For example, acetaldehyde (Ace), Acr, Cro, and HNE can bind to the DNA guanine residue to from the highly mutagenic 1,N^2-propano-2'-deoxyguanosine (1,N^2-propanodGuo, 1,N^2-PdG) adduct [259,277,279-281]. However, few studies, to our knowledge, have identified the specific aldehydes produced by AFB1-induced LPO and their contribution to cancer development in human or animals. An early study demonstrated a dose-dependent production of MDA and conjugated dienes in rat liver homogenates treated with AFB1; and this production was prevented by a pre-treatment of the cells with antioxidants and iron chelators [282]. The study also demonstrated that the aldehydes produced accumulate in the cellular microsomes, nucleus, and mitochondria and damage them. A subsequent study conducted in the same laboratory further demonstrated that lipid peroxidation in hepatocytes increased with increasing doses of AFB1 of 10-100 mM [283]. Although the latter study provided evidence for the implication of hydrogen peroxide and hydroxyl radical as the main AFB1-genrated ROS responsible for LPO, it provided no indication about the nature of the resulting aldehydes. Conversely, a recent study showed that AFB1-induced OS in human hepatocytes (HepG2) released Acet and Cro with a subsequent formation of the highly mutagenic cyclic α-methyl-γ-hydroxy-1,N^2-propano-deoxyguanine (meth-OH-PdG) DNA adduct [284]. Interestingly, the study demonstrated that OS plays more prominent role in AFB1-mediated mutagenicity than does AFBO, as was substantiated by the following findings: (i) AFB1 treatment

of the human hepatocytes generated more than 30 times higher levels of meth-OH-PdG adducts than AFB1-N^7-gua, (ii) like AFB1, Acet and Cro targeted the hotspot codon 249 on *p53* gene to cause G→T transversion mutations, but they had higher preferences for the site than AFBO, and (iii) the DNA repair of the meth-OH-PdG lesions produced by Ace and Acr was significantly slower than that observed with AFBO-derived DNA lesions. Indeed, strong inhibition of both BER and NER by LPO-generated aldehydes and its enhancement by the accompanying epigenetic modifications is well documented [285-287]. In addition to reduced DNA repair of the meth-OH-PdG-mediated lesions, methylation of the cytosine on codon 248 (-*CGG-) was shown to promote the adduct formation on the adjacent codon 249 of tumour suppressor *p53* gene [284]. Moreover, the concomitant production of 8-OH-dG by ODD, discussed above, may enhance the epigenetic effect, as this adduct was shown to increase cytosine methylation at the -*CpG- islands [288]. On the other hand, it is well established that reactive LPO-induced aldehydes are more mutagenic than the free radicals and the highly reactive AFBO [272]. Indeed, reactive aldehydes can act remotely from the site of their formation, contrary to the short-lived and highly instable free radicals and epoxides. This may also account for a reason why AFB1 can mediate cancer in organs distant from the liver where the aflatoxin is activated and metabolized [196]. Despite the shortage in studies related to aflatoxin-mediated OS, the available data clearly suggest that OS plays more important roles in aflatoxin toxicities than presently assumed. In fact, the above-mentioned study of Weng, Lee, Choi [284] suggests that the role of OS has been undermined so far and should be further investigated. The high efficacy of selenium in preventing HCC onset in chicken provides additional evidence for the implication of OS in tumorigenicity of aflatoxins, since this trace mineral acts primarily by enhancing the anti-oxidative capacity of cells [289-290]. It also provides an additional hint for the toxicity of aflatoxins, which do formation of the reactive epoxide upon metabolic activation by the liver cytochrome enzymes, such as AFB2 and AFG2 (Figure 1) and other AFB1 metabolites [32] whose furan ring does not have the double bond between C8 and C9 carbons.

6 Immunotoxicity

Increased frequency and severity, and prolonged healing of infectious diseases, in addition to decreased vaccination efficacies provided evidence that aflatoxins disrupt both innate and acquired/adaptive immunity [291-295]. The general mechanisms of AFB1immunotoxicity via AFBO is presented in Figure 2. It can be seen from the figure that AFBO interacts with immunocompetent cells throughout the body to affect their proliferation and/or production of immune response mediators, thereby disrupting the innate and adaptive immunity. Although most studies to illustrate these mechanisms have been carried out on animals, the immunotoxicity of AFB1 has also been substantiated in vitro on human cell lines and in case-control studies in highly exposed regions, e.g., Ghana [293,296-298]. However, few studies to our knowledge have investigated the immunotoxicity of aflatoxins other than AFB1 or its combination with other mycotoxins [299-302]. Meanwhile, there has been a general agreement that low or moderate concentrations of AFB1 have no or a marginal immunotoxicity, and that cell-mediated immunity (CMI) is more susceptible to aflatoxins than humoral immunity [293,300,303-304].

A concentration of 60 µg AFB1/kg feed given ad libitum to weanling pigs for 33 days had no noticeable effects on the counts of different types of leukocytes and lymphocytes, or on antibody and cytokine titres, while the highest concentration tested of 180 µg/kg feed had only moderate effects on leukocyte counts and Tumour necrotizing factor (TNF)-α [301]. This appears to be especially relevant that young pigs are among the most susceptible hosts to aflatoxins [305]. In rats, oral administration of 100 µg AFB1/kg bw once a week for five weeks inhibited only slightly the proliferation of lymphocytes with no significant effect on related secretions of cytokines, chemokines, and immunoglobulins in the serum; a ten-fold higher dose of 1 mg AFB1/kg bw could only increase numbers of $CD8^+$ (cytotoxic T lymphocytes), while various other immunological parameters remained unchanged [306]. Similarly, feeding rats ad libitum on feed contaminated with AFB1 at different levels (0.01-1.6 mg/kg feed) for longer periods (up to

40 weeks every other 4 weeks) exerted significant effects on the immune response only at the highest concentrations of 0.4 and 1.6 mg/kg after 12 weeks of exposure or longer [307]. Nonetheless, other studies suggested that lower concentrations of aflatoxins and shorter durations of exposure can still alter the immune response. For example, feeding rats with diet containing five to 75 μg AFB1/kg bw for five weeks [308], or dosing mice intraperitoneally with 25 or 50 μg AFM1/kg bw five days per week for 4 weeks [299] have altered their immunity in time- and dose-dependent manner.

On the other hand, the higher susceptibility of CMI compared with the humoral immunity is well documented, as has been reviewed previously [293,298]. For example, dosing rats with 0.6 mg AFB1/kg bw had no significant effect on IgM titre, while ten-fold lower dose (0.06 mg/kg bw) could inhibit lymphocyte proliferation [309]. Also, ingestion of 0.1 or 1 mg AFB1/kg bw did not alter anti-ovalbumin IgE and IgG antibody production in rats mesenteric lymph nodes despite their significant action on the proliferative activity on B and/or T lymphocytes [306].

Regarding the mode of immunomodulation of the immune function by aflatoxins, most of the available data suggest that they mainly exert suppressive effects; however, in vitro and in vivo studies have demonstrated that they can also dysregulate the immune response via immunostimulatory effects [310-311].

6.1 Immunosuppression

Immunosuppression is manifested by the destruction of the physical barriers as a first line defence against invaders (pathogens and toxins), the inhibition of proliferation and function of immunocompetent cells, or the decrease in complement system activity, thereby interfering with both innate and adaptive immunity [293,298].

6.1.1 Innate immunity

Destruction of physical barriers such as the skin and the intestinal epithelial cells with a consequent impairment of the barrier function against microbial and toxin invasions has been demonstrated in vivo and in vitro. Contact of AFB1 with the skin of different animal was reported to elicit various types of lesions spanning from the formation of intra-epidermal vesicles to squamous cell carcinoma [302,312-314]. Feeding pigs for 28 days on feed contaminated with mixtures of aflatoxins (AFB1, AFB2, AFG1, and AFG2) produced crusting and skin ulceration on the snout, lips, and buccal commissures [302]. Aflatoxins have also been demonstrated to disrupt the integrity and function of the mechanical barrier of intestines by interfering with the cell cycle progression or by destroying the intestinal epithelial cells and the tight junctions (TJs) that cement them together. Administration of 0.6 mg AFB1/kg diet to broilers for 3 weeks stalled the cell cycle at the G2/M phase causing reduction in the height jejunum and in the ratio of villus height/crypt, thereby impairing their function as a selective barrier [315]. These findings were recently corroborated by feeding broiler chicken with feed containing 0.6 mg AFB1/kg for up to 21 days and monitoring structural and functional changes in the small intestine [316]. The study showed various structural and histopathological injuries similar to those described above regarding the increased depth of villi with decreased height and area [315], in addition to other histopathological alterations in the small intestine, including mitochondrial vacuolation and loss of cristae, reduced numbers of the absorptive cell goblets and the junctional complexes. Such changes alter dramatically the barrier function of the intestine to interfere not only with nutrient absorption, but also with the innate immune response as a protective means against the invasion of pathogens or toxins. Indeed, increased gut permeability was induced in broilers fed on feed contaminated with 1.5 mg AFB1/kg for 20 days [317]. Lower concentrations of aflatoxins AFB1 (16.3-134 μg/kg feed) and AFB2 (3.15-23.6 μg/kg feed) orally administered to broilers for up to 42 days disturbed the cell cycle progression and apoptosis causing histopathological lesions with different severities in thymus and bursa fabricius where T and B lymphocytes undergo maturation, respectively [318]. At the molecular level, aflatoxins have been demonstrated to alter the mechanical, chemical, and

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immune barriers that protects the intestinal mucosa against various external threats. In vitro exposure of human cell line CacO-2 to 1-100 μM of AFB1 for 48 h decreased the trans-epithelial electrical resistance (TEER) with consequent increase in the paracellular permeability and decrease in the viability [319]. The study related the latter effects to downregulation of the transcription of three constitutive proteins of the TJs, claudin-3, claudin-4, and occludin. In a similar study, CacO-2/TC7 cells exposed to AFM1 (3.2 and 33 nM) for 24 h showed reduced the TEER of the monolayer and accelerated transport of the aflatoxin through it, meanwhile, the TJs and their constitutive proteins remained intact [320]. Likewise, the selective permeability of CacO-2 cells was disrupted upon exposure to different amounts of AFM1 (0.2 to 20 μM) for 48 h [321]. The latter study associated the permeability disruption to reduction of TEER, down-regulation of the expression of structural TJ proteins (claudin-3, claudin-4, occludin, zonula occludens-1), and decrease in the levels of p44/42 mitogen-activated protein kinase (MAPK) involved in cell death or cell survival. Other AFB1-induced structural disturbances of the gastro-intestinal tract that alter immune functions with special focus on broiler chicken have been thoroughly reviewed previously [311].

Effects of aflatoxins on immune cells that play key roles in the innate immunity, such as monocytes, macrophages, dendritic cells (DC), and natural killer (NK) cells to restrict their viability, function, or genetic expression of cytokines and chemokines is well documented (Figure 2). Exposure of broilers to AFB1 was reported to repress the transcription of toll-like receptors (TLR) TLR-2, TLR-4, and TLR-7, indicating a suppressive effect on the innate immunity where these receptor proteins are involved in the recognition of external invaders by sentinel cells, e.g., macrophages and dendritic cells, as a key step to trigger this type of immune response [316]. AFB1 at low doses 10 ng/mL were also reported to reduce the antigen-presenting activity of porcine dendritic cells, although this reduction could not be associated with down-regulation of the expression of TLRs or specific cytokines [322]. Moreover, aflatoxins AFB1, AFB2, and/or AFM1 were reported to reduce viability, proliferation, cytotoxicity, and phagocytic activity of macrophages as well as their expression of cytokines, e.g., TNF-α, IL-1, and IL-6, and the inducible nitric oxide synthase (iNOS) that mediate intracellular killing of pathogens in phagocytosis [300,323-327]. Recently, AFB1 was demonstrated to dysregulate the innate immune function mediated by autophagy and external trap formation in M1-type macrophages responsible for inflammatory reaction, which is triggered by the secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12, IL-23, [310]. Pre-treatment of human monocytes with as low concentration as 0.1 pg AFB1/mL for 24 h prior to incubation with *Candida albicans* for 30 min at 37°C has impaired significantly their phagocytic and killing activities towards the pathogenic yeast [328]. In vitro reduction of chemotactic response to bacterial chemoattractant factor, a phagocytic stimulatory mediator, was demonstrated on neutrophils harvested from the blood of piglets that had been suckling milk contaminated with AFB1, AFM1, and AFG1 [329]. Intraperitoneal administration of AFM1 to mice at doses of 25 and 50 mg/kg bw reduced significantly its phagocytic activity against *E. coli* [299]. As regards the effects of aflatoxins on the proliferation and cytotoxic activity of NK cells, conflicting data are available in the literature. While mice gavage with 0.03-0.7 mg AFB1/kg inhibited cytolysis of YAC-1 cells by NK cells in BALB/c [330], the same concentrations or even higher (24 mg/kg bw) had no such effect in C57B1/6 mice [331]. However, a significant reduction in the proliferative and cytotoxic activities of human NK cells was demonstrated in vitro upon incubation of the cells with 0.005-0.05 ng AFB1/mL [332]. Phagocytic and cytotoxic activities of dairy cow neutrophils against *Staphylococcus aureus* and *Escherichia coli* were also dramatically hampered upon exposure to low doses of AFB1 (0.01, 0.05 and 0.5 ng/mL) for 18 h, which was ascribed to the depletion of neutrophil cytosol from ROS, playing pivotal role in the killing process of pathogens during phagocytosis, rather than affecting the viability of the neutrophils themselves [333].

The complement system as a crucial component of the innate immunity that activates phagocytosis of infectious pathogens, was shown to be inhibited by aflatoxins in various animals. Dosing guinea pigs *per os* daily with 30 μg AFB1 or greater amounts for 20 days decreased the complement activity [334], while a dose of 10 μg has no noticeable effect on these innate immune mediators [335]. A decrease in the

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complement activity was also demonstrated by feeding trials in cattle and poultry at different threshold levels [303,311,336]. Concentrations ranging between 0.11 to 0.21 mg AFB1/kg feed were shown to impair both classical complement pathway and alternative pathway of complement activation (APCA) in duckling [337]. However, according to Valtchev, Koynarski, Sotirov [304], feeding ducklings with AFB1 at doses of 0.5 or 0.8 mg/kg feed for 40 days had a stimulatory effect on the APCA in the first 15 days followed by suppressive effect during the next days of the experiment. Yet, the effect of aflatoxins on the complement system may depend largely on the host, as no significant change in the serum hemolytic activity (CH_{50}) was recorded in rabbits exposed to as high level as 24 mg/kg feed for 28 days [338]. AFM1 was demonstrated to reduce significantly the complement system in Balb/c mice receiving a dose of 25 or 50 mg/kg bw, as evidenced by a decrease in CH_{50} using rabbit anti-sheep red blood cells (RBC) IgG antibodies and sheep RBC [299].

6.1.2 Adaptive immunity

Suppression of adaptive/acquired immunity upon exposure to aflatoxins is well established indicating the increased vulnerability of exposed hosts to infectious agents, as well as the reduced or failed protection of vaccination [295,339-340]. The latter effect has been demonstrated in poultry by epidemiological studies correlating aflatoxin exposure to poor protection by vaccination against Newcastle disease [311] and infectious bronchitis [341]. Similar suppressive effect of immunisation was reported in pigs where vaccination failed to protect them against *Erysipelothrix rhusiopathiae* when given AFB1-contaminated feed, contrary to a control group receiving aflatoxin-free feeds [340]. Also, decreased proliferation, activation, and/or function of lymphocytes, as the main immune cells that promote adaptive immunity, has been demonstrated in humans and animals. A dose- and time-dependant apoptotic effect was observed on human peripheral blood lymphocytes incubated with different doses of AFG1 (3.12-2000 µg/L) for different times (2-72 h) [342-343]. In vitro exposure of human lymphocytes to AFB1 at concentrations of 5-165 µM induced a dose-dependent increase in numbers of apoptotic and necrotic lymphocytes, with an evident rise in cell necrosis starting from 50 µM (~15.6 mg/L) at 24 h of incubation [344]. In vitro exposure of human lymphoblastoid Jurkat T-cell line to AFB1 or AFM1 at 3–50 µM for up to 72 h inhibited the proliferation of the T cells in a dose-dependent manner starting at 15 µM, but did not cause their apoptosis or necrosis [345]. According to the same study, AFB1 and AFM1 increased significantly the expression of IL-8 involved in innate immunity, while the adaptive immunity remained unaffected as suggested by unchanged levels of INF-γ and IL-2 cytokine compared to negative control cells incubated in the absence of aflatoxins. A concentration of 10 mg AFB1/L or greater inhibited the differentiation of mitogen-induced T and B lymphocytes in cattle with a consequent impairment of both T-cell dependent and T-cell independent humoral immunity, and hence immunoglobulin production [346]. Up-to 10 mg AFB1/kg feed was required to supress IgG and IgA production by B lymphocytes and to restrict the humoral response against *Salmonella* and rabbit red blood cells in chicken [339,341,347]. Also, intraperitoneal administration of 50 µg AFM1/L for four weeks (five times a week) to mice did not affect the concentration of IgM in the blood serum [299]. In addition, a dose of 1.8 mg AFB1/kg feed given to pigs for 18 days did not stimulate anti-ovalbumin IgG production in the serum despite the induction of mitogenic activity of lymphocytes, indicating that this dose of the aflatoxin specifically supresses the activation of B lymphocytes but not their proliferation [294]. Concentrations below 0.5 mg AFB1/kg feed did not affect antibody responses to *Pasteurella multocida*, *Salmonella pullorum* and Newcastle disease in broiler chicken and turkey [303]. Although it is now well established that disruption of humoral immunity requires higher aflatoxin dosage than does CMI, no consensual threshold levels that alter CMI or humoral immunity have been reached so far. Such levels vary widely depending on the species, the age, the gender, and the route of administration. In poultry, doses of 0.4 and 1.0 mg AFB1/kg feed are the most accepted such thresholds for CMI and humoral immunity, respectively [311].

Suppression of adaptive CMI has been studied on laboratory animals, mainly poultry and rodents, demonstrating a decrease in numbers of different subsets of T-cell lymphocytes and cytokines they produce, as key elements in this type of immune response [311,348]. Adaptive CMI suppression by aflatoxins was also evidenced by decreased delayed-type hypersensitivity (DTH) in different animals at concentrations ranging between 0.3 and 1.0 mg/kg feed [311,349]. DTH was significantly delayed/decreased in broilers and turkeys receiving AFB1 dose of 0.2 mg/kg feed or higher for 33 days [347]. Conversely, a subsequent study showed that a two-fold higher dose did not affect DHT in broilers, which was, by contrast, significantly reduced when the same dosage consisted of a combination of AFB1 and AFB2 [350]. A one-day-old broilers receiving 0.6 mg AFB1/kg feed for three weeks displayed reduced proportions of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T-cell subsets as well as the transcription of different cytokines in the birds intestines, thereby impeding adaptive CMI [348], where these T-cell subsets and some of the inhibited interleukins, e.g., IL-2 and INFγ, play crucial role [351]. Proliferation and cytokine production by splenic helper T lymphocytes ($CD4^+$) involved in acquired cellular immunity were also reduced in rats given AFB1 doses ranging between five and 75 µg/kg bw for five weeks [308]. Similar effects were induced by AFM1 in mice dosed intraperitoneally with at 25 or 50 µg/kg bw five days per week for 4 weeks, where suppression of acquired CMI was evidenced by a decrease in DTH and related T lymphocytes subsets ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and CD49b) as well as the interleukins they produce, e.g., INFγ, IL-10, and IL-4 [299]. In humans, elevated levels of AFB1, as estimated by the concentrations of AFB1-albumin adduct in the serum, were highly correlated with the decrease in lymphocyte subsets $CD3^+$ and CD19+ bearing the D69 activation marker (i.e., $CD3^+CD69^+$ and $CD19^+CD69^+$), and $CD8^+$ T-cells which play a central role in vaccination and immune response against pathogens [297]. The latter results suggest that AFB1 impairs acquired CMI in humans and decreases their resistance to infections, consistent with the reported accentuation of impaired activation of $CD8^+$ and $CD4^+$ T lymphocytes in human immunodeficiency virus (HIV)-positive Ghanaian patients [352].

It should be pointed out, however, that humoral immunity and CMI whether they are acquired or innate cannot always be separated. For example, any dysregulation of the proliferation and/or TLRs expression in dendritic cells will have direct repercussions on innate and adaptive immunity, as these antigen-presenting cells are key intermediates between both types of immune response [327,353].

6.2 Immunostimulation

Regarding the immunostimulatory effects, there is increasing evidence that aflatoxins illicit a biphasic immune response with a stimulatory action in the first phase and suppressive action in the second [311]. According to Valtchev, Koynarski, Sotirov [304], exposure to low doses of aflatoxins for short periods stimulates the immune system, while exposure to higher doses for longer periods exerts immunosuppressive effects. For example, the transcription of TLR-2 and TLR-4 was up-regulated in human myeloid dendritic cells (DC) exposed to environmentally relevant doses of AFB1 (1 or 2 µg/L) for 2 to 24 h [296]. Up-regulation of TLR expression have been demonstrated in different immune cells from different organs as a means to sense very low levels of aflatoxins [209,296,327,333]. A single dose of 663 µg AFB1/kg bw given to mice by gavage up-regulated the production of both the inflammatory cytokine IFN-γ and the anti-inflammatory cytokine IL-4 after 5 days of the ingestion [354]. The authors attributed such an unusual reaction to the activation of innate immune cells after a short time of administration of a high dose in a single shot, as a first step preceding the trigger of an adaptive response. Intermittent intake of AFB1 simulating the actual situation was also reported to result in an alternation of suppressive and stimulatory/compensatory effects upon exposure and resting (aflatoxin-free diet) periods, respectively [307]. Despite the conflicting data and lack of consensus regarding the cytokine types induced in response to aflatoxin exposure, unnecessary up-regulation of the immune response stimulates the production of tissue-damaging inflammatory molecules and free radicals leading to chronic inflammation, cancer, and nervous system degenerative diseases [209,268]. Moreover, low levels of a mixture of aflatoxins (AFB1,

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AFB2, AFG1, and AFG2) increased the antigen-presenting capacity of dendritic cells that stimulate T-cell proliferation, which was suggested to breakdown the immunological tolerance and increase host susceptibility [322].

7 Teratogenicity

Exposure of pregnant females or birds to aflatoxins can affect embryos *in utero* or in fertilized eggs, respectively, producing various adverse health effects and different pathological gestation/incubation outcomes [355]. In mammalians, systemic blood circulation in highly exposed mothers conveys aflatoxins or their toxic metabolites to foetuses, as has been substantiated in highly exposed pregnant women from African and Asian countries, as well as in animals. Indeed, aflatoxins and/or biomarkers derived thereof, e.g., aflatoxin metabolites, and aflatoxin-DNA and aflatoxin-albumin adducts, were detected in the cord blood of the foetus or in both foetal cord and maternal blood samples [356-360]. Accordingly, it was concluded that aflatoxins or their metabolites in pregnant women are transmitted to the foetus and metabolized through the same pathways as in adults [360]. Therefore, pregnancy of highly exposed mothers is prone to various outcomes, including foetal growth restriction, foetal loss, and premature birth. Growth restriction has been documented in humans and animals where an inverse relationship between the birthweight and the amounts of appropriate biomarkers in the cord blood has been extensively demonstrated [357,361-363]. Conversely, few studies have related high-aflatoxin exposure of pregnant women to stillbirth, while studies on the association of high aflatoxin intake by pregnant women with premature birth and foetal loss are either non conclusive [363] or lacking [362]. On the contrary, decrease in live birth and litter size, impairment of organ development, and skeletal anomalies in offspring have been demonstrated in animals given aflatoxins at daily doses ranging between 0 (nil) and 100 μg/kg bw, which was explained by the binding of aflatoxins to the DNA and the hindrance of protein synthesis [355,364-368]. This view that can apply to humans, as aflatoxins bind to human DNA in the same way, but it remains to be clinically demonstrated.

In addition to the above-mentioned adverse health effects, aflatoxin-rich diet of pregnant females affects their health and expose their foetuses to indirect consequences with congenital abnormalities. For example, up-regulation of maternal pro-inflammatory cytokines and/or downregulation of anti-inflammatory cytokines induce systemic inflammation that impairs the placental growth and causes its insufficiency ultimately leading to poor foetal growth, miscarriage and stillbirth, or prematurity [301,356,361]. Also, the cytotoxic activity of aflatoxins induces anaemia in mothers by lysing red blood cells or interfering with nutrient, e.g., iron, selenium, and vitamins, absorption with consequent poor foetal growth and/or prematurity [369-371]. The association of anaemia and high aflatoxin intake, as determined by AFB-albumin adduct in the mothers' serum, was demonstrated in cross-sectional study on Ghanaian women [363]. On the other hand, the association of anaemia to red blood cell lysis by aflatoxins was demonstrated in vitro and in animal species dosed with 0.5 to 1.0 mg/kg bw [372-375]. However, it appears that the environmentally relevant levels of aflatoxins remain below the doses that can elicit red blood cell lysis in humans. Conversely, there is a lack of evidence on the association between inflammation-induced anaemia in pregnant women and their exposure to aflatoxins. As matter of fact, there are many gaps in the knowledge of doses, mechanisms, and outcomes of exposure to aflatoxins in pregnant women that require more attention and rigorous scientific approaches to be clearly understood and eventually avoided to ensure safe pregnancy and birth.

8 Other Adverse Health Effects of Chronic Exposure to Aflatoxins

In addition to the major toxicological effects reviewed above, aflatoxins exert various other adverse health conditions with overlapping mechanisms and risk factors. These include malnutrition diseases (faltering and stunting), retarded physical and mental maturity, reproduction and sexuality, and nervous system diseases (neurodegenerative diseases and neuroblastoma) [180,376-378]. However, most of the

latter effects have been scarcely investigated to cover the main pertaining aspects from applied and mechanistic standpoints. Therefore, further studies are needed for clearer insights on these issues to have an accurate and realistic opinion on the risk they may pose to the public health. This section addresses malnutrition and neurodegenerative diseases, which have been relatively well studied

8.1 Aflatoxins and malnutrition

Malnutrition is probably one of the above-mentioned aspects that has received the most attention due to its impact on the childhood in many developing countries, where children are already facing food shortage to ensure balanced and healthy growth, and hence be well prepared to adulthood as active and productive individuals. Exposure to aflatoxins exacerbates such poor nutritional status by interfering with the absorption of vitamins and minerals, as is the case for vitamins A, C, and E, and selenium, which not only deprives children/consumers from these essential micronutrients, but also increases their susceptibility to aflatoxins that they normally detoxify owing to their inherent antioxidant or CYP P450 inhibitory activities [290,355,379]. As a result, exposed children may experience growth disorders from the gestational stage as discussed above to the adulthood with stunting and retarded physical and mental maturity [380]. Indeed, in African countries, growth faltering among children below 5-years old was correlated with chronic exposure to high levels of aflatoxins when they rely on local agricultural products, e.g., maize, peanut, and derivatives as staple foods [381]. On the other hand, severe protein energy malnutrition (PEM) diseases, Kwashiorkor and marasmic kwashiorkor, have been associated with chronic exposure to high levels of dietary aflatoxins in different African countries [382-385]. However, since all the relevant studies were conducted in poor household environments where children are invariably fed on local agricultural products with poor nutritional and hygienic quality and limited availability, PEM could be due to the limited access to enough nutritious foods rather than aflatoxin intake. To address this particular issue, a study has been conducted on malnourished Soudanese children with Kwashiorkor, marasmic kwashiorkor, or marasmus. The results of the study revealed that a group of kwashiorkor and marasmic kwashiorkor children had significantly higher levels of AFB1 and its derivative aflatoxicol in their sera and urine compared with a group of malnourished children with marasmus and a group of age-matched normally nourished children [384]. Accordingly, the authors concluded that kwashiorkor is definitely correlated with high chronic exposure to aflatoxins as either secondary to liver damage or an aetiological factor of the disease, which remains to be further substantiated by appropriately designed future studies [384].

8.2 Aflatoxins and neurodegenerative diseases

In addition to the classically known adverse health effects of aflatoxins, there is increasing body of evidence that chronic exposure to aflatoxins can also be responsible for neurodegenerative disorders. The AFBO and ROS generated by CYP450 enzymes and aflatoxin-induced oxidative stress, respectively, react with functional macromolecules in neuronal brain cells where they inhibit lipid and protein synthesis to induce their degeneration [386]. Aflatoxins were also reported to disrupt the structure and function of mitochondria of brain cells that impedes the oxidative phosphorylation leading to cell apoptosis [387]. In addition, the detection of aflatoxins in brain tissues of kwashiorkor-deceased child and their association with Rey's disease (cerebral edema and neuronal degeneration) is a strong indication that they can cross the brane blood barrier and infiltrate the nervous system that they degenerate [209,376]. Although scarce, epidemiological studies have indeed demonstrated the neurotoxicity of aflatoxins in humans and animals. In a recent study, rats dosed with 1/600th their LD_{50} dysregulated the levels of biochemical biomarkers of the oxidative stress indicative of neurodegenerative disorders, which were corroborated by histopathological and immunohistochemical tests showing vasodilation, necrosis and astrocytes gliosis [376]. In addition to the oxidative stress, aflatoxins induce neurodegenerative disorders by dysregulating

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the immune response of immunocompetent cells to create a proinflammatory conditions in the central nervous system [209].

9 Acute toxicity

The ingestion of aflatoxins at high levels in a single dose or repeatedly for a short period of time induces acute intoxication in humans and animals with typical symptoms, including jaundice, lethargy, nausea, edema, hemorrhagic necrosis of liver tissues, bile duct hyperplasia, and eventually death (10-60%) subsequent to severe liver damage [388]. Although there is no consensus on the specific dose of aflatoxins that triggers acute toxicity in humans, it is well established that such a dose is highly variable depending on many factors, including the age, gender, health and nutritional status, presence or absence of underlying factors (e.g. chronic viral hepatitis, alcoholism, smoking, cirrhosis, exposure to hepatotoxic microcystins); and it is lowest in youngsters, as substantiated by the highest death rates of this age-group in aflatoxicosis outbreaks [180,185,187]. A rough estimation of the acute dose of AFB1 concerned a case report on a-15-years old Ugandan child weighing 36 kg who has been eating AFB1-contaminated cassava on a daily basis until he died by liver failure [191]. The authors calculated the likely cumulative amount of cassava that caused the child's death to be 3.1 kg contaminated with 1.7 mg/kg, corresponding to a total dose of 146 µg/kg bw that he had eaten in 22 successive days before death. However, these calculations are very approximate, as they were based on the lethal dose of AFB1 to monkeys, and on the assumption that the AFB1 concentration in cassava, determined retrospectively after the death, was constant throughout the whole period of intake preceding the death. Nevertheless, the outcome of these calculations, is in accordance with an estimation of the world health organization (WHO) based on records of aflatoxicosis outbreaks worldwide and in vitro tests, which considers that regular consumption of food contaminated with 1 mg AFB1/kg or higher for a short period causes acute intoxication in humans [178]. According to the same report, daily consumption of food contaminated with AFB1 at a dose of 0.02-0.12 mg/kg bw over 1 to 3 weeks causes a life threatening aflatoxicosis. Furthermore, the cumulative lethal dose in humans was suggested to vary from 10 to 20 mg for adults and 3 mg for children [186], which is also consistent with the estimated total dose of ~5.3 mg ingested in 22 days by the Ugandan teenager [191]. Nonetheless, deliberate ingestion of 5.5 mg chemically pure AFB1 over two days and 35 mg over two weeks in suicide attempts by an adult women from the USA was reported to cause no serious aflatoxin-related injuries at admission to the hospital for mild symptoms and even 14 years later [192]. Although difficult to explain, this could be due to her overall well-balanced nutritional status, age, and gender, since well-nourished adult females are less susceptible to aflatoxins than males of similar health and nutritional status [389-390].

In animals, the lethal dose varies greatly among species, as suggested by the wide variation in their LD_{50} values ranging between 0.3 and 18.0 mg/kg bw [391], although values as low as 0.2 mg/kg bw or as high as 60 mg/kg bw were occasionally reported (Table 2). Animals like ducks, sheep, turkeys, dogs, pigs, and rats are the most susceptible, whereas monkeys, chickens, mice, and ruminants the most resistant [144,354]. The higher susceptibility of the first group of animals was explained by their ability to metabolize rapidly AFB1 via the phase II metabolism driving towards the formation of aflatoxin-albumin adducts [305]. In a study on the impact of an orally administered single dose of AFB1 to mice, 0.66 mg/kg bw induced severe tissue injury 5 days after the ingestion [354]. In poultry, the AFB1 doses that killed all tested birds varied between 0.8 and 4.0 mg per animal, with turkeys being the most sensitive (0.8 mg) and gooses the most resistant (4.0 mg), whereas no death was observed in chicken at the highest doze of 4.0 mg [391].

The mechanism of acute aflatoxicosis is poorly understood, although many authors refer to the interaction between aflatoxins and macromolecules (proteins, phospholipids, and nucleic acids) with a consequent formation of various adducts, which in turn interferes with physiological and structural functions of the macromolecules. In particular, aflatoxin-protein adducts have been the most frequently associated with the acute intoxication, as this blocks protein synthesis, especially enzymes involved in vital

functions, such metabolic pathways, protein synthesis, DNA replication and repair, and immune response (Figure 2). Additionally, there is increasing evidence that aflatoxin-phospholipid adducts and ROS-induced LPO are the main responsible for the disruption of integrity and functions of membranes of the cells, mitochondria, and endoplasmic reticulum [202,208], as depicted in Figure 2. Moreover, severe DNA fragmentation upon exposure to high doses of aflatoxins is another major effect in acute aflatoxicosis (Figure 2), as was observed in testicular tissues of mice injected with a daily dose of 20 µg AFB1/kg bw for 21 days [248]. However, a recent study on the acute toxicity of AFB1 in poultry, suggested that aflatoxin-dihydrodiol (AF-dhd) is the main responsible for the acute aflatoxicosis, as the pivotal metabolite leading to the formation of aflatoxin-albumin adducts [305]. According to the authors, AF-dhd derives from aflatoxin-exo 8,9-epoxide and forms the aflatoxin-albumin adducts via aflatoxin-aldehyde bypassing the formation of aflatoxin-dialcohol of the detoxification pathway [1]; and the more rapidly and abundantly AF-dhd is formed, the higher is the mortality rate. Moreover, the metabolism of $AFB2_a$ as a dietary contaminant or as an AFB1-phase I metabolite was also suggested to be involved in acute toxicity; apart from the formation aflatoxin-albumin adducts, $AFB2_a$ is was also reported to bind covalently with cellular proteins and phospholipids yielding lipid- and protein-adducts possibly leading to acute aflatoxicosis [202].

It should be pointed out, however, that chronic exposure to low doses of aflatoxins can produce similar effects as those observed in acute aflatoxicosis; however, their effects can be mitigated by detoxifying phase II enzymes and cellular antioxidant defence mechanisms, or by DNA repair to prevent mutations, as discussed above (section 5.1). Alternatively, these effects accumulate progressively with continuous exposure to low doses to, ultimately, evolve in liver cancer as the typical outcome of chronic exposure. Therefore, acute aflatoxicosis may result from an abrupt accentuation of most or all of the above-mentioned damages in a short time when the dose is too high. An overwhelming amount of aflatoxins can overcome the detoxifying capacity of the cell and drives the metabolism of the toxins towards the production of toxic metabolites causing severe DNA damage, disruption of cell cycle progression, DNA fragmentation, metabolic disorders, cytotoxicity, and tissue necrosis eventually leading to organ failure (Figure 2) in a short period. This may hold especially true as the adverse aflatoxin effects are cumulative [392-393]. For example, FAPy-DNA adduct burden that triggers tumorigenesis in rats was estimated to be 1 adduct per 250,000 nucleotides, i.e., 40,000 adducts/cell [394], which can either accumulate progressively with chronic exposure or be reached in short time in case of exposure to abnormally high doses of AFB1.

10 Conclusions

Aflatoxins are widespread highly toxic contaminants that require further research to clarify many essential aspects for better knowledge of their toxicity patterns and occurrence in foods and feeds in order to address adequately their adverse effects on public health and economy. Particular attention should be paid to improvement of the situation in developing countries where crops, such as peanut, maize, sorghum, and sunflower, prone to aflatoxin contamination are grown in favourable agroclimatic zones (hot and humid) to aflatoxin production. Continued high contamination of produce originating from endemic regions is a major hurdle to international trade and to food security, as this does not affect only local populations, but may extend to other parts of the world by either exporting highly contaminated goods or restricting their marketability, which in turn contribute to increase their prices and limit accessibility to poor social strata. Unfortunately, despite the efforts made in these regions to reduce foods and feeds contamination with aflatoxins, the most recent data gathered in this review suggest that there is no such trend, and the incidence and contamination levels vary from one year to another depending mainly on the meteorological conditions, with highest contaminations and incidence recorded in rainy seasons generally proceeded by dry seasons. Yet, the use of atoxigenic strains of *Aspergillus flavus* in the newly developed

biocontrol technology to colonize endemic AEZ and displace the aflatoxigenic strains appears to be a promising intervention that should be encouraged and further investigated.

The information reviewed herein reflects the scarcity or lack of information on aflatoxins other the major ones (AFB1, AFB2, AFG1, and AFG2), whose occurrence in foods and feeds, and roles in toxicity have so far been overlooked. In addition, despite the intensive work that has been carried out on the toxicity mechanisms of aflatoxins for more than five decades, it is clear that the extent and nature of health disorders are not well understood due to their high complexity and the intricate and overlapping risk factors, some of which may be confounding factors.

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