CO-EXPOSURE OF AFLATOXIN AND FUMONISIN IN NIGERIAN MAIZE AND THE NON-CARCINOGENIC RISK OF AFLATOXIN IN SOUTH-WEST NIGERIAN CHILDREN AND ADULTS

By

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ABSTRACT

CO-EXPOSURE OF AFLATOXIN AND FUMONISIN IN NIGERIAN MAIZE AND THE NON-CARCINOGENIC RISK OF AFLATOXIN IN SOUTH-WEST NIGERIAN CHILDREN AND ADULTS

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Aflatoxins are secondary fungal metabolites that frequently contaminate food crops such as maize and peanuts. They are well known to cause liver cancer; however, multiple studies have also found aflatoxin to be immunotoxic. Studies also show that aflatoxin and fumonisin (another mycotoxin) may have synergistic toxicological effects. This dissertation determines the prevalence of these two mycotoxins in Nigerian maize and maize products, explores if lactic acid bacteria (LAB) fermentation effectively reduces these mycotoxins in a popular commercially produced maize cereal in Nigeria and evaluates the immunotoxicological risk of aflatoxin in southwest Nigerian children and adults.

Our hypothesis was that aflatoxin and fumonisin occur and co-occur at multiple stages of the southwest Nigerian maize value chain. We analyzed the occurrence and co-occurrence of aflatoxin and fumonisin from harvest to postharvest storage to processing and final food and feed products in the marketplace (chapter 2). Some of the samples collected form farmers' storage contained alarming levels of total aflatoxins (> 400 ppb) which could potentially cause acute aflatoxicosis in humans. About 52% of the samples exceeded the Nigerian standards for aflatoxins and 13% of the samples contained fumonisin levels that exceeded the US regulatory limit. The co-occurrence was found to be at multiple stages along the maize value chain.

Next, we examined if lactic acid fermentation significantly reduces aflatoxin and fumonisin concentrations in commercially produced ogi which is a popular cereal produced from maize in

Nigeria (chapter 3). Ogi is consumed by potentially vulnerable populations such as young children and the elderly or ill, so it is important to consider the risk of mycotoxins in this food. Our hypothesis was that lactic acid bacteria (LAB) fermentation can significantly reduce mycotoxin level in commercially produced ogi. We have analyzed the levels of aflatoxin and fumonisin before and after LAB fermentation using LC-MS/MS and found it to reduce both mycotoxins after processing. However, the reduction was statistically significant only for fumonisins ($P<0.05$).

As aflatoxin is a genotoxic carcinogen, international risk assessment bodies have never established a non-carcinogenic tolerable daily intake (TDI) for aflatoxin since there is no threshold assumption made for cancer. There is substantial evidence in the literature that aflatoxin may have immunotoxin effects. Hence, we have determined a range of TDI of aflatoxin (0.017 to 0.082 µg/kg BW/day), based on the existing data surrounding aflatoxin and biomarkers of immune suppression (chapter 4).

Finally, we have conducted a quantitative risk assessment on immunosuppressive endpoint of aflatoxin in southwest Nigerian children and adults based on our calculated TDI and dietary aflatoxin exposure through maize and groundnut consumptions (chapter 5). Our hypothesis was that the rural populations in southwest Nigeria are at great risk from aflatoxin-induced immunosuppression. Our risk assessment indicates a reasonable risk of aflatoxin-induced immunosuppression in children residing in the rural settings of southwest Nigeria. The risk is comparatively lower in children living in the urban sector with a chance of possible risk. Adults living in rural sector are also at possible risk. On the other hand, the adult population residing in the urban sector does not seem to be at risk from aflatoxin-induced immunosuppression. Taken together, the results presented in this dissertation advance understanding of the exposure, risks and impacts of mycotoxins in high-risk populations in Southwest Nigeria.

This dissertation is dedicated to my loving parents (Tapan Kumar Saha & Jhunu Saha) for their limitless love, sacrifices and for letting me move thousands of miles away to a different country to achieve my goals. I also dedicate this to my fiancé for being a constant source of support and motivation throughout my PhD journey.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

A.	*Aspergillus*
ADD	Average Daily Dose
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFP1	Aflatoxin P1
AFQ1	Aflatoxin Q1
AF-alb	Aflatoxin B1 albumin adduct
AFB1-FAPy	Aflatoxin B1-formamidopyridine adduct
BMD	Benchmark dose
BMDL	Benchmark dose lower bound
BMDS	Benchmark Dose Software
BMI	Body Mass Index
BMR	Benchmark Response
C	Carbon
C_{ave}	Average concentration
CYP	Cytochrome P450

DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
ESI	Electrospray Ionization
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
F.	*Fusarium*
FAO	Food and Agricultural Organization
FB	Fumonisin B
FDA	United States Food and Drug Administration
GH	Growth Hormone
GSH	Glutathione
GST	Glutathione-S-transferase
h	Hour (s)
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
HPLC	High-performance liquid chromatography
HQ	Hazard Quotient

IARC	International Agency for Research on Cancer
IBV	Infectious Bronchitis Virus
IFN	Interferon
IgA	Immunoglobulin A
IITA	International Institute of Tropical Agriculture
IL	Interleukin
IP	Intraperitoneal
IR_{ave}	Average Intake Rate
JECFA	Joint FAO/WHO Expert Committee on Food Additives
KW	Kruskal-Wallis
LADD	Lifetime Average Daily Dose
LGA	Local Government Area
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of detection
MSU	Michigan State University
MWW	Mann-Whitney-Wilcoxon
NK	Natural killer
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
OH	Hydroxyl

OTA	Ochratoxin A
OVA	Ovalbumin
ROS	Reactive oxygen species
SON	Standard Organization of Nigeria
STAT6	Signal transducer and activator of transcription
T-2	T-2 toxin
TDI	Tolerable Daily Intake
TGFβ	Transforming growth factor beta
TLC	Thin Layer Chromatography
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UF	Uncertainty Factor
UPLC	Ultra Performance Liquid Chromatography
US-EPA	United States Environmental Protection Agency
WBC	White Blood Cell
WHO	World Health Organization
ZEA	Zearalenone

CHAPTER ONE: Background

1. Aflatoxins and fumonisins: two of the major agro-economical food-borne mycotoxins and their public health impacts

Mycotoxins are toxic chemical compounds that are secondary metabolites produced by filamentous fungi, or molds, which contribute to serious risks for human and animal health (Ji *et al.*, 2016). Multiple adverse health effects of mycotoxins are observed in both humans and animals which include carcinogenicity, teratogenicity, immune toxicity, neurotoxicity, hepatotoxicity, nephrotoxicity, reproductive and developmental toxicity, gastrointestinal disturbances (McKean *et al.*, 2006; Pleadin *et al*., 2019). Mycotoxins can contaminate a variety of important agricultural and food products in the field, during storage or transportation, depending on the product's moisture content, water activity, temperature, pH, relative air humidity, food matrix composition, the amount of physical damage, and the prevalence of mold spores (Pleadin *et al*., 2019). Due to the fungal infection of crops, mycotoxins can end up in the human food chain either by direct consumption or when used as livestock feed (Marin *et al.*, 2013). *Aspergillus*, *Fusarium*, and *Penicillium* are the fungal genera to which the major fungi producing mycotoxins belong (Sweeney and Dobson, 1998; Marin *et al*., 2013).

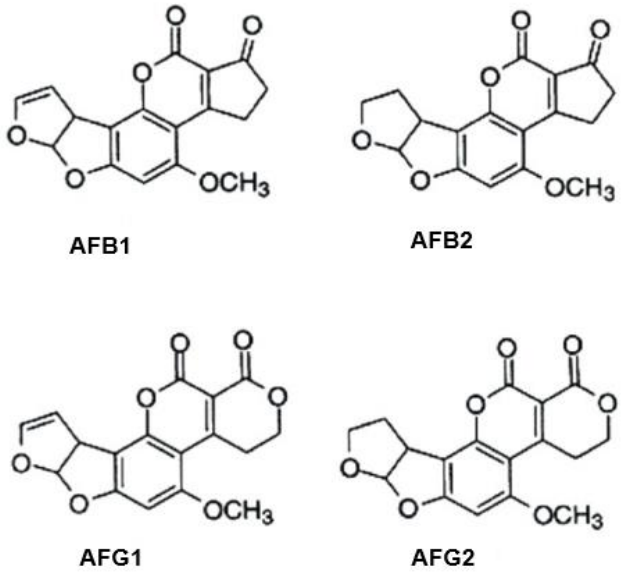
While *Aspergillus* and *Penicillium* species commonly grow under storage conditions. *Fusarium* species often infect crops in the field and spread in the plant (Tanaka *et al*., 1988; Bennett and Klich, 2003). People living in the developing nations are more susceptible to the health risks associated with mycotoxins because these are frequently produced in tropical and subtropical conditions and the staple diets in many developing countries include crops which are frequently contaminated with mycotoxins (Bhat and Vasanthi, 2003). Currently, more than 300 mycotoxins have been identified, however, only six are regularly found in food and feedstuff, that contribute to food safety problems globally (Alshannaq and Yu, 2017); these include aflatoxins (AF),

fumonisins, ochratoxins A (OTA), patulin, zearalenone (ZEA), and trichothecenes (deoxynivalenol (DON) and T-2 toxin). Aflatoxins and fumonisins are two most common mycotoxins with widespread occurrence in cereal crops and feeds which concern both public and animal health worldwide (Bruns, 2003; Nishimwe *et al.*, 2019).

1.1 Aflatoxins

Aflatoxins belong to one of the predominant mycotoxins in food produced by secondary metabolism of the species *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins were first discovered in 1960 soon after an epidemic of "Turkey X disease" in England where more than 100,000 turkeys suddenly became ill and died in the course of a few months (Blount, 1961). Aflatoxins are produced in wide variety of food crops such as cereals (maize, rice, barley, oats and sorghum), groundnuts, pistachios, walnuts, almonds and cottonseeds (Wu *et al*., 2014; Alshannaq and Yu, 2017). Factors that influence aflatoxin production are drought stress, rainfall, insect damage, crop genotype and poor agricultural practices (Khlangwiset *et al*., 2010; Wu *et al*., 2011). Aflatoxins show great resistance to conventional treatments that are applied to process food and feedstuffs, such as pasteurization, sterilization and other thermal applications (Rustom, 1997). Hence, preventive measures need to target the contamination of crops throughout the production chain, mainly during pre- and post-harvest maneuvers (Ismail *et al*., 2018). The four major types of aflatoxins are aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) (structures illustrated in Figure 1). Among these four types of aflatoxins, AFB1 is the most toxic and also is the form most commonly found in food, therefore, it is the most studied mycotoxin due to its toxic and genotoxic potency (Van Egmond *et al.*, 2007; Wu *et al.*, 2014). Its hydroxylated metabolite aflatoxin M_1 (AFM_1) can be found in milk and other dairy products from dairy animals that have consumed AFB_1-contaminated feed.

Figure 1: Chemical structures of the four major naturally produced aflatoxins: AFB1, AFB2, AFG1 and AFG2



Note: The figure was adapted from "Risks of Environmental Genotoxicants" by S. Attia and G. Harisa, 2016, *Environmental Health*, 139. Copyright 2016 by Gamal Harisa.

Over the last 60 years, aflatoxin exposure has been associated with multiple adverse health outcomes. The International Agency for Research on Cancer (IARC) has classified "naturally occurring mixes of aflatoxins" as a Group 1 human liver carcinogen (IARC 2002). The risk of aflatoxin-related liver cancer becomes 30 times higher for individuals who are simultaneously infected with chronic hepatitis B virus (HBV) infection (JECFA 1998; Wu *et al.*,2013). Aflatoxin consumption at high doses is associated with acute aflatoxicosis (poisoning resulting from aflatoxin ingestion), acute liver damage, edema, and even death (FDA 2004). Aflatoxin is also associated with growth impairment in children, pregnancy loss, premature birth, and immunotoxicity (Bondy & Pestka 2000; Khlangwiset *et al.*,2011; Wild *et al.*,2015; Smith *et al.*,2017).

Consumption of maize and groundnuts are the major sources for human exposure to aflatoxins (≈ 4.5 billion people are exposed to aflatoxins (Wild and Gong, 2010) since the consumption rates of these foods are high worldwide and maize and groundnuts are highly susceptible to *Aspergillus* infection; Approximately 25% of the world's crops are estimated to be contaminated by aflatoxins (Strosnider *et al*., 2006). Hence, significant efforts are required to minimize the aflatoxin contamination in foodstuffs, especially in developing nations in order to reduce its impacts on public health.

1.1.1 Mechanism of Action for aflatoxin-induced toxicity

Aflatoxins contribute to various toxicological effects with different mechanisms, most of which are not fully explained yet. In order to exert its hepatocarcinogenic effect, AFB1 is bio-transformed by cytochromes P450 (CYP) that are present in the liver, to form AFB1-8,9-exo-epoxide and AFB1-8,9-endo-epoxide. AFB1-8,9-exo-epoxide is highly reactive and it binds to DNA to form a predominant 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1–N7-Gua) adduct (Iyer *et al*., 1994; Kensler *et al.*,2011; Obuseh *et al*., 2011). These DNA adducts (if not repaired before DNA replication) can interact with the guanine base of the DNA to cause mutation in the p53 tumor suppressor gene resulting in hepatocarcinogenesis (Wang and Groopman, 1999; Obuseh *et al*., 2011). AFB1 can be bio-activated by different CYP450 isozymes depending on the host, the organ, and the sub-cellular component (Benkerroum, 2020). In humans, the microsomal CYP1A2, 3A4, 3A5, 3A7, 2A3, and 2B7, the hepatocytic 3A3, and the lung CYP2A13 are the major isozymes responsible for AFB1 bioactivation in the corresponding organs (Echizen *et al*., 2000; Nelson *et al*., 2004). Among these, the major CYP enzymes involved in human aflatoxin metabolism are CYP3A4 which forms the exo-epoxide and another metabolite called AFQ1, and CYP1A2 which forms some exo-epoxide and high proportions of endo-epoxide and AFM1 (Wild and Turner,

2002). If not excreted through urine and milk, AFM1 can also be epoxidized to reactive AFM1-8,9-epoxide and bind to DNA to form AFM1-N7-guanine adduct (Jager *et al.*, 2011). The epoxides can also bind to the tripeptide glutathione (GSH) which is an antioxidant, and undergo detoxification reactions facilitated by glutathione-S-transferase enzymes, and form aflatoxin-mercapturate which is readily excreted in urine (Turner, 2013). The other metabolites of AFB1 (such as: AFQ1, AFP1) and AFB2, AFG1, and AFG2 are not effectively epoxidized and so they are non-genotoxic and less toxic compared to AFB1 (Wild and Turner, 2002). In animals and insects, depending on the species and the organ where they are produced, CYP1A1, 1A, 1A2, 2A5, 2A6, 3A, 3A4, 3A13, and 321A1 CYP450 isozymes are reported to be responsible for the bioactivation of AFB1 (Benkerroum, 2020).

Even though the mutagenicity of aflatoxins has been mostly attributed to the formation of aflatoxin-N7-gua DNA adducts, there is also evidence that AFB1 can induce DNA damage by oxidative stress due to the release of reactive oxygen species (ROS) during AFB1 metabolism, leading to oxidative stress (Bedard and Massey, 2006). This oxidative stress can further act directly on DNA to cause oxidative DNA damage or can also form by-products from lipid peroxidation of membrane phospholipids (Klaunig *et al.*, 2009). The ROS can also bind to nitrogen bases and deoxyribose moieties of the DNA to generate more DNA adducts (Klaunig *et al.*, 2009).

Other toxic health effects associated with AFB1 are also primarily attributed to the formation of AFB1-8,9-exo-epoxide. Previous studies have reported the reactive AFB1-8,9-exo-epoxide to be potentially responsible for aflatoxin-induced immunomodulation. For instance, the AFB1-8,9-exo-epoxide metabolite can interrupt DNA-dependent RNA polymerase activity which can inhibit synthesis of RNA and proteins (Raney *et al.*,1993). This reduction in protein synthesis might directly or indirectly affect the proliferation/differentiation of immune cells and interleukin

production and therefore disrupt the communication between immune system mediators affecting both innate and adaptive immunity (Dugyala & Sharma 1996, Benkerroum, 2020). The mechanism of acute aflatoxicosis is not well elucidated, however, it is referred to the interaction between aflatoxins and macromolecules, such as proteins, phospholipids, and nucleic acids. This can lead to formation of several adducts that can affect macromolecular physiology and functions and inhibit production or function of enzymes which have important roles in metabolic pathways, DNA repair and replication, protein synthesis and immune response (Benkerroum, 2020). The cell membrane integrity and functions of cells, mitochondria, and endoplasmic reticulum might also be disrupted by the aflatoxin-phospholipid adducts and the by-products from lipid peroxidation (Marin and Taranu, 2012; Rushing and Selim, 2017).

1.1.2 Contribution of aflatoxins to hepatocellular carcinoma (HCC)

The reaction of AFB1-8,9-exo-epoxide and guanine residues produces the AFB1-N7-guanine adduct, which forms an opened ring structure making a stable AFB1-formamidopyridine adduct (AFB1-FAPy) on the guanine residue of DNA. The AFB1-formamidopyridine adducts induce DNA lesions which have been known as the main precursors for genotoxic and carcinogenic effects of AFB1 (Groopman *et al*., 1981; Chawanthayatham *et al.*,2017). The guanine residue can also undergo depurination releasing free AFB1-N7-guanine which is then excreted in the urine and is often used as a biomarker for aflatoxin exposure (Vidyasagar *et al*., 1997; Smela *et al.*, 2001; Egner *et al.*, 2006)**.** Hepatocellular carcinoma (HCC) or liver cancer is one of the leading causes of mortalities (more than 600,000 people per year) in the world (Ferlay *et al*., 2004). It is estimated, 4.6–28.2% of all global HCC cases may be attributable to AFB1 exposure (Liu and Yu, 2010). Early epidemiological studies in Uganda and Kenya showed that high levels of aflatoxin contamination in food was prevalent in regions that had high incidence of liver cancer, which

corresponded with AFB1's hepatocarcinogenic properties in laboratory experiments (Alpert *et al*., 1971; Peers and Linsell, 1973). Moreover, co-exposure to chronic hepatitis B virus (HBV) infection and aflatoxin exposure play important role in occurrence of HCC in developing countries. There is evidence that the risk of HCC is greatly enhanced with combination of AFB1 exposure and HBV infection, far above either factor individually, indicating a synergy between AFB1 and HBV (Kew, 2003; Loomba *et al*., 2013). In developing countries, HBV is a serious and frequent illness that is responsible for 80% of HCC cases globally (Kucukcakan and Hayrulai-Musliu, 2015). If an individual is exposed to chronic HBV and AFB1 together, the risk of developing HCC increases up to 30 times higher (Liu and Wu, 2010), which has been a public health concern for a long time.

1.1.3 Aflatoxin and growth impairment

Aflatoxin to exposure is identified as one of the major risk factors for causing childhood stunting. Over the last few decades, several studies have indicated that exposure to AFB1 is associated with growth impairment in both humans and animals (Gong et al., 2004; Khlangwiset et al., 2011; Watson et al., 2018).

Several studies indicate that higher aflatoxin exposure in pregnant women can be associated to poor birth outcomes (Maxwell et al., 1989; Smith et al., 2017). There is considerable evidence that fetuses and newborns can be exposed to aflatoxins in utero and through breast milk when the mothers get exposed to aflatoxins (Maxwell et al., 1989; Wild et al., 1991; Abdulrazzaq et al., 2003; Mahdavi et al., 2010; Ghiasain and Maghsood, 2012). A Kenyan study investigating 125 pregnant women found that more than half of the mothers had detectable levels of aflatoxin biomarkers in the blood and 37% of the cord blood samples were also positive for aflatoxin biomarkers (De Vries et al., 1989). This study also found the mean birth weight of girls born to

pregnant women whose blood tested positive for aflatoxin, was significantly lower than those born to mothers with no detectable levels of aflatoxin in the blood (De Vries et al., 1989). A study in the United Arab Emirates detected AFM1 in blood of 100% (43 of 43) of neonates born with low birth weights, but only in 55% (68 of 123) of neonates who had normal birth weights, indicating a strong negative correlation between AFM1 levels and birth weights (Abdulrazzaq et al. 2004). A Gambian study including 138 infants for a year, found significant negative associations between aflatoxin exposure in mothers during pregnancy and height and weight gain of their infants (Turner et al., 2007). Continuous exposure to aflatoxins post-weaning can further affect the development in children which was demonstrated by Gong et al. (2002 and 2003). In a cohort study of 480 children (age: 9 months to 5 years) in Benin and Togo, aflatoxin B1 albumin adducts (AF-alb) was found in the blood of 99% of the children with higher levels in post-weaning ages (>3 years old) (Gong et al., 2002, 2003). The studies found dose-response relationships between AF-alb levels and stunting parameters. The mean AF-alb levels were 30-40% higher in stunted children who were stunted compared to the non-stunted children (Gong et al., 2002, 2003). These studies indicated that weaning is a critical stage for exposure to aflatoxin in children and aflatoxin contamination in diets of post-weaned children should be minimized to prevent growth impairment.

The mechanism of how aflatoxin leads to growth impairment is not well elucidated, however, many different mechanisms are proposed by different authors based on in vivo studies. AFB1 exposure led to suppression of hepatic insulin-like growth factor-1 (IGF-1) mRNA expression, liver injury and resistance to hepatic growth hormone (GH) in rats; liver damage and changes in GH signaling could be a potential mechanism of AFB1-associated growth impairment (Knipstein et al., 2015). Some studies (both human and *in vivo*) also linked aflatoxin-induced intestinal disease

or enteropathy to be a contributor of growth impairment because the intestinal tissue damage or infiltration of pathogens interfere with vitamins and mineral absorption and may increase inflammation (Maresca and Fantini, 2010; Obuseh et al., 2011; Smith et al., 2017).

Growth impairment in children is a major public health issue that affects millions of children in the world, especially in developing nations. Stunted children often develop long-term developmental and cognitive problems later in life and are more susceptible to infectious diseases (Ricci et al., 2006). Therefore, it is essential to abate aflatoxin exposure in both pregnant women and children in order to prevent its potential long-term effects.

1.1.4 Aflatoxins and immunotoxicity

In low-income nations, the majority of childhood deaths result from infectious disease. Aflatoxin contamination of staple foods such as maize and peanuts is common throughout sub-Saharan Africa; this results in chronic dietary exposure to aflatoxin in many populations (Xu *et al*., 2018). There are limited epidemiological studies that have explored the effects of aflatoxins on the immune system, however, the limited studies indicate that aflatoxin exposure may contribute to impairments in both cellular and humoral immunity (Turner *et al.* 2003; Jiang *et al*., 2005). However, the mechanisms by which aflatoxins result in immunomodulating effects have not been clearly determined. Previous studies have reported the reactive –8-9 epoxide to be potentially responsible for aflatoxin-induced immunomodulation. For instance, the –8-9 epoxide metabolite can interrupt DNA-dependent RNA polymerase activity which can inhibit synthesis of RNA and proteins (Raney *et al.*, 1993). This reduction in protein synthesis might directly or indirectly affect the proliferation/differentiation of immune cells and interleukin production and therefore disrupt the communication between immune system mediators affecting both innate and adaptive immunity (Dugyala and Sharma, 1996; Benkerroum, 2020).

Multiple studies have indicated that aflatoxin exposure can impair innate immune cells including macrophages, neutrophils and NK cell-mediated functions (Reddy and Sharma, 1989; Neldon-Ortiz and Qureshi, 1992; Silvotti *et al.*, 1994; Cusumano *et al.*, 1996; Bonomi and Cabassi, 1997; Moon *et al*.,1999a, Cheng *et al*., 2002; Meissonnier *et al.*, 2008; Mohsenzadeh *et al*., 2016). Aflatoxin exposure was found to decrease T- and B-lymphocyte activities, which are the key cellular components of the adaptive immune response (Richard *et al*., 1978; Reddy *et al*., 1987; Hinton *et al*., 2003; Jiang *et al*., 2015). Numerous *in vivo* studies also demonstrated that aflatoxin exposure can alter the levels of cytokines produced by both innate and adaptive immune cells (Hinton *et al*., 2003; Meissonnier *et al.*, 2008; Li *et al*., 2014; Qian *et al.*, 2014; Jiang *et al*., 2015; Ishikawa *et al*,. 2017; Shirani *et al*., 2018; Wang *et al*., 2018).

High aflatoxin exposure was found to be associated with more rapid HIV disease progression; possibly due to reduced $CD4^+$ and $CD8^+$ T-cell counts in individuals who are already infected with HIV (Jiang *et al.*, 2008). There is substantial evidence in the literature that aflatoxin exposure may increase the risk of immune system dysfunction by disruption of both innate and adaptive immunity.

It is estimated that around three million children die every year, mainly in low- and middle-income countries, from vaccine preventable infectious diseases (Duclos *et al*., 2009). Even though vaccination ranks among the most cost-effective tools in public health, the effectiveness of it can be influenced by many environmental factors, hence not all children around the world develop the same protective immune response to the same vaccine (Githang'a *et al*., 2019a; Githang'a *et al*., 2019b). There is evidence that exposure to aflatoxin can occur during critical developmental stages of the immune system (Khlangwiset *et al*., 2011; Smith et al., 2017). Some studies exploring effects of aflatoxin on effectiveness of vaccination have indicated that aflatoxin exposure may

impair vaccine response (Batra *et al*., 1991; Azzam and Gabal, 1998; Meissonier *et al.,* 2008; Yunus and Böhm, 2013); this means even if people receive vaccines in Sub-Saharan Africa or other high-risk areas with high exposure to dietary aflatoxins, their response to vaccines may be impaired. This is a particularly critical outcome; as in developing countries, vaccine-preventable infectious diseases are known to be a major cause of child mortality. Also, dietary aflatoxin exposure is more common in developing countries, which increases the likelihood of impaired vaccine responses in the vulnerable children in these populations.

1.2 Fumonisins

Fumonisins are water-soluble secondary toxic metabolites, first isolated in 1988, produced by *Fusarium verticillioides* and *F. proliferatum* (Demir *et al*. 2010). Fumonisins mainly contaminate maize and maize-based products but can also be found in rice, sorghum, wheat bran, soybean meal, and poultry feed (Stockmann-Juvala and Savolainen, 2008). Among many fumonisin analogues identified so far, the most frequently found are fumonisins B1 (FB1), B2 (FB2) and B3 (FB3). FB1 is the most prevalent and found at higher concentrations (about 70%) in contaminated food (Rheeder et al. 2002). Environmental factors such as temperature, moisture and post-harvest practices influences the production of fumonisins in crops (Blandino et al. 2009; Paterson and Lima 2010).

Fumonisins are associated with various animal and human adverse health effects (Visentin *et al.,* 2012). They were initially discovered in horses through its association with equine leukoencephalomalacia outbreak and later also linked with causing porcine pulmonary edema (Marasas, 2001). Fumonisins are classified as Group 2B possible human carcinogen by IARC (IARC, 2002). It has been associated with causing esophageal and liver cancers (Sun *et al*., 2007,

2011). Dietary fumonisin exposure in pregnant mothers has been linked to neural tube defects in infants (Missmer et al., 2005; Marasas *et al*., 2004). In the last decade, studies have associated fumonisin exposure with growth impairment in children (Kimanya *et al*., 2010; Shirima *et al.*, 2015; Chen *et al*., 2018a; Chen *et al*., 2018b). Therefore, several nations have set regulatory standards for fumonisins in food products. The European Union (EU) has set maximum limits of 200 ppb in baby foods, 800 ppb in breakfast cereals, and 4000 ppb in unprocessed maize (Scott, 2012). The US-FDA regulates total fumonisin ($FB_1 + FB_2 + FB_3$) levels at 2000 ppb for processed maize and 4000 ppb for raw maize in human food and 5000-10000 ppb in animal feed (FDA, 2001).

Fumonisins exposure and consumption can be reduced in several ways. Cleaning damaged or moldy corn kernels can reduce fumonisin concentrations. Since fumonisins are water soluble, cooking in alkaline water and getting rid of the liquid afterwards can lower the concentration in food. Even though fumonisins are heat-stable, baking, frying and extrusion cooking at high temperatures can partially reduce them (Bullerman and Bianchini, 2007; Burns et al., 2008; Kaushik, 2015). However, it is not well understood if these thermal processes actually reduce concentrations of fumonisins due to thermal decomposition because fumonisins can actually form covalent bonds and bind to macromolecules such as, sugar, protein or lipids and be modified upon thermal treatment and processing (masked fumonisin) (Streit et al., 2013).

1.2.1 Adverse effects of fumonisins

The human health effects of fumonisins are unresolved, nonetheless studies have associated consumption of maize contaminated with fumonisins to esophageal and liver cancers (Ueno *et al.* 1997; Marasas, 2001; Fandohan *et al*., 2005). Fumonisins are also associated with neural tube defects (Cortez-Rocha *et al*. 2002; Humpf and Voss, 2004; Missmer *et al.*, 2006). During 1990-

1991, high prevalence of neural tube defects among Mexican-American women along the Texas-Mexico border were reported, which was attributed to the frequent consumption of corn tortillas that might be contaminated with high levels of fumonisins (Missmer *et al.*, 2006). FB1 can cause toxicity to the liver and to the kidney in many laboratory and farm animal species (Voss *et al.*, 2007). FB1 is also associated with toxicity in the cardiovascular system in pigs and horses (Smith *et al.*, 1996; Smith *et al.*, 2002). Over the last two decades, studies have also found association between fumonisin exposure and child growth impairment (Kimanya *et al*., 2010; Shirima *et al.*, 2015; Chen *et al*., 2018a; Chen *et al*., 2018b).

1.2.2 Mechanism of action for fumonisin-induced toxicity

FB1 has a primary amino group which can inhibit ceramide synthase resulting in disruption of the de novo biosynthesis of ceramide and sphingolipid metabolism (Chuturgoon et al., 2015). The inhibition of ceramide synthase by fumonisins prevents the formation of ceramide from sphinganine and fatty acyl-CoA leading to increased tissue and serum concentrations of sphinganine, sphingosine, and their 1-phosphate metabolites (Ahangarkani et al., 2014). This mechanism of toxicity caused by Fumonisin B are reflected on protein kinase activity, cell proliferation and differentiation, cell death (apoptosis), carcinogenicity and involvement of lipid peroxidation (Soriano *et al*., 2005). A possible mechanism for fumonisin-associated neural tube defects could be that the disruption in sphingolipid metabolism by FB1 could affect the uptake of folate in pregnant women and cause neural tube defects in their babies, as folate deficiency is a major risk factor (Marasas *et al*., 2004).

2. Co-exposure of fumonisins with aflatoxins

While there is strong evidence that the individual exposure to aflatoxins and fumonisins can cause adverse health effects in both humans and animals, over the last 2 decades both *in vivo* and *in vitro* studies have indicated that the co-exposure of these two mycotoxins may have additive and synergistic effects in the development of liver cancer initiated by aflatoxin. A broiler chicken study indicated that co-exposure to these mycotoxins had additive effects on body weight, liver structure and immunological response (Tessari et al., 2006). Oral doses of pure aflatoxin and fumonisin in mice resulted in increased relative spleen weight and increased oxidative stress (Abbes et al., 2016). A rat study indicated that sequential exposure to aflatoxin and fumonisin showed synergistic effects on liver enzymes (alanine transaminase and aspartate transaminase) implying that fumonisins may act as a promoter for aflatoxin-initiated liver cancer (Qian et al., 2016). Mitchell *et al*., (2014) studied the effects of co-exposure of these mycotoxins in male Fischer 344 rats and found the AFM1 excretion in urine was reduced by almost 65% in co-exposed animals compared to the AFB1 alone-exposed animals (Mitchell *et al*., 2014). The AFB1–albumin adduct levels were significantly higher in the co-exposed group compared with rats given only AFB1 (1100 vs 600 pg adduct/mg albumin, respectively). This study results indicate that FB1 may induce increased production of the reactive AFB1 - 8,9-epoxide intermediate, which could potentially increase the risk of hepatocarcinogenicity of AFB1.

In 2016, JECFA, the Joint FAO/WHO expert committee on food additives explicitly studied the relationship between aflatoxin and fumonisin and their co-exposure in causing adverse effects in humans, during their 83rd meeting. It was concluded that there is not enough data to know for sure if co-exposure contributes to human diseases, however, since AFB1 is genotoxic and fumonisin, has potential to induce regenerative cell proliferation, the co-exposure still remains a

concern especially in developing countries where the co-exposure of these mycotoxins is high (JECFA, 2016).

3. Impacts of mycotoxin on human health and economic implications in developing countries

In developing countries, especially sun-Saharan African countries, mycotoxin contamination of staple crops, such as maize and groundnuts, causes significant postharvest losses, negative impacts on health, as well as economic welfare (Lewis et al., 2005; Mutegi et al., 2013). It is estimated that the global food crop contamination by mycotoxins is 25% (WHO, 1999). In developing nations, the contamination of mycotoxins is significantly more prevalent compared to the developed countries due to many reasons which include: 1) lack of strict regulatory mechanisms, 2) climatic and crop storage conditions being favorable to fungal growth and mycotoxin production, 3) diets being less diverse, 4) populations relying on subsistence farming or on local market food that are not appropriately regulated and so forth. The socio-economic status of majority of residents of sub-Saharan African countries also makes them liable to consume more of mycotoxin contaminated products either directly or at various points in the food chain. There is plenty of evidence that shows populations in sub-Saharan Africa are chronically exposed to high levels of mycotoxins, especially aflatoxins and fumonisins which pose many different health risks including cancer, growth impairment, immunosuppression etc. This is particularly concerning among children because more than half of the global under 5 deaths occur just in sub-Saharan Africa (UNICEF, 2020). Since mycotoxin associated stunting and growth impairment during early age may contribute to increasing long-term disease burden and also make children more vulnerable to infectious diseases, it is extremely crucial to control mycotoxin exposure in these children. Moreover, hepatitis B and C virus infections are common in sub-Saharan African countries, which

multiplicatively increases the risk of liver cancer from aflatoxin exposure (Liu et al., 2012; Wu *et al.*,2013; Qureshi *et al.*, 2014).

Mycotoxins are one of the most important contributors to economic losses from food and feed in developing countries, especially in Sub-Saharan Africa (Udomkun *et al*., 2017). Losses due to rejected shipments of exported crops due to mycotoxin contamination above regulation standards, and lower prices for poorer quality of the crop can devastate the export markets in developing countries. Aflatoxin contaminated feed can affect animal health leading to major economic losses due to decreased performances and reproductive disorders (Stepman, 2018).

Exposure to mycotoxins needs to be immediately addressed in developing countries in order to improve human health and economy. Specific interventions can be implemented to overcome this major problem, such as, introduction of genetically modified crops, use of bio-control agents, better control of the fungal growth by using of fungicides and pesticides, better post-harvest storage practices, insects control measures during storage, fermentation (Stepman, 2018).

CHAPTER TWO: The occurrence and co-occurrence of aflatoxin and fumonisin along the maize value chain in southwest Nigeria

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Abstract

Aflatoxin and fumonisin are two major foodborne mycotoxins: toxic chemicals produced by fungi that contaminate food commodities including maize, a staple food in sub-Saharan Africa. Aflatoxin causes liver cancer, and is associated with acute liver toxicity and immunotoxicity; while fumonisin is associated with neural tube defects in infants and esophageal cancer. Both mycotoxins have been associated with child growth impairment. Previous studies suggest that co-occurrence of these mycotoxins may have potentially synergistic toxicological effects. Despite health risks associated with co-occurrence of these mycotoxins, no study has examined their cooccurrence along key food supply chains in Africa. This study is the first report that examines the occurrence and co-occurrence of aflatoxins and fumonisins along the maize value chain in Nigeria. All samples were analyzed using LC-MS/MS. About 52% and 21% of the samples had aflatoxin levels above the Nigerian and US standards for human food, respectively. Though no regulatory limits exist for fumonisin in Nigeria, 13% of the samples contained fumonisin levels higher than the US regulatory limit. Aflatoxin levels can become dangerously high in maize stored four months or longer. Adequately addressing mycotoxin risk requires consideration of the entire maize value chain and associated value chains for food production.

Key words: Aflatoxin, Fumonisin, Co-occurrence, Value chains, Maize, Nigeria

1. Introduction

Aflatoxins and fumonisins are two major groups of mycotoxins produced by Aspergillus and Fusarium fungi respectively. These mycotoxins frequently contaminate maize, mainly in countries with high temperature and humidity (Paterson and Lima, 2017). They have been implicated in multiple adverse human and animal health effects (Eze*et al*., 2018; Alshannaq and Yu, 2017; Wu *et al*., 2014; Shephard, 2008). In recent years, international organizations such as the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization and World Health Organization recognize the importance of the co-occurrence of aflatoxins and fumonisins in maize, because of potentially interacting toxicological effects (JECFA, 2017, 2018). But the nature of this co-occurrence in actual food for human consumption, and associated health effects, are still largely unstudied.

"Naturally occurring mixes of aflatoxins" are classified as a Group 1 human liver carcinogen by the International Agency for Research on Cancer (IARC, 2002). Aflatoxin contributes to causing hepatocellular carcinoma (HCC); additionally, the risk of aflatoxin-related HCC is multiplicatively higher for individuals who also have chronic hepatitis B virus (HBV) infection (JECFA, 1998; Wu *et al*., 2013). High doses of aflatoxin can result in acute aflatoxicosis, characterized by liver failure, edema, and even death. Aflatoxins are also associated with growth impairment in children (Wild *et al*., 2015; Khlangwiset *et al*., 2011). A recent study has found that aflatoxin exposure is significantly higher in stunted children compared to non-stunted children in Nigeria (McMillan *et al*., 2018). Aflatoxin exposure may also be associated with pregnancy loss and premature birth (Smith *et al*., 2017) and immunotoxicity (Bondy and Pestka, 2000). Fumonisins were discovered initially through its association with equin leukoencephalomalacia outbreak and further investigations also found its association with causing porcine pulmonary

edema (Marasas, 2001). Fumonisin is now classified as a Group 2B possible human carcinogen (IARC, 2002). It has been associated to a limited extent with esophageal and liver cancers (Sun *et al*., 2007, 2011). Dietary fumonisin exposure in pregnant mothers has been linked to neural tube defects in infants (Missmer *et al*., 2005; Marasas *et al*., 2004). In the last decade, studies have associated fumonisin exposure with growth impairment in children (Kimanya *et al*., 2010; Shirima *et al*., 2015; Chen *et al*., 2018a; Chen *et al*., 2018b).

Several animal and in vitro studies of aflatoxin-fumonisin co-exposure indicate additive or synergistic effects on the development of precancerous lesions or liver cancer (JECFA, 2018). A study in broilers indicated that co-exposure to aflatoxin and fumonisin had additive effects on body weight, liver structure and immunological response (Tessari *et al*., 2006). In a recent study, oral doses of pure aflatoxin and fumonisin in mice resulted in increased relative spleen weight and increased activity of enzymes that lead to oxidative stress, in a potentiating manner (Abbes *et al*., 2016). In a rat feeding study, exposure to pure aflatoxin or fumonisin alone or sequentially showed effects on body weight to be less than additive, but effects on some liver enzymes were synergistic; supporting the theory that fumonisins may act as a promoter for aflatoxin-initiated liver cancer (Qian *et al*., 2016). Taken together, these studies suggest the possibility of increased hepatocarcinogenicity from co-exposure to aflatoxins and fumonisins (JECFA, 2018; JECFA, 2017; JECFA, 1998). The exact mechanism on how aflatoxins and fumonisins interaction leads to toxicity is not very clear yet. However, a previous rat study suggest that co-exposure may result in a decreased excretion of AFB1 through the urine and increased levels of serum AFB1 –albumin adduct that forms the reactive AFB1 -8,9-epoxide intermediates which ultimately leads to hepatocarcinogenicity (JECFA, 2017; Mitchell *et al*., 2014). Recent studies also show that chronic exposure to high levels of fumonisins may result in inhibition of ceramide synthase (Riley *et al*.,

2015). This, in addition to increased sphingosine kinase activity, could enhance the development and progression of several human tumors (Espaillat *et al*., 2015); and possibly promote the tumorigenic potential of AFB1 initiated DNA damage (JECFA, 2017).

Previous toxicological studies show solid evidence about the adverse human health effects from the consumption of aflatoxins. According to a dose response approach, it is estimated that 25,200–155,000 cases of liver cancer globally may be associated to aflatoxin exposure every year (Liu and Wu 2010). Even though the evidence for adverse health effects from fumonisin consumption in humans is currently not very conclusive, there are concerns that it may contribute to various serious adverse health outcomes including cancer and birth defects (WHO, 2018). Developing countries such as Nigeria are more at risk due to the climatic and crop storage conditions favoring the fungal growth and mycotoxin production. In addition, maize is often mixed with other commodities in the production of food and feed. These all create many opportunities for aflatoxin and fumonisin contamination during the production, handling, and storage of maize products.

Furthermore, the prevalence of chronic hepatitis B viral infection in Nigeria is also very high: about 12.2% (Olayinka *et al*., 2016). Since dietary exposure to aflatoxins among Nigerians is very likely, is an important concern for the country. The Standards Organization of Nigeria (SON) has set standards for maximum total aflatoxin concentrations in maize for 4 μg/kg (SON, 2008). However, fumonisin levels are not known to be regulated in food and feed in Nigeria. Maize is an essential crop for food security in Nigeria as well as an industrial crop (USDA, 2014). Maize in Africa is frequently contaminated with both aflatoxins and fumonisins (Kimanya *et al*., 2008). Nigeria, Africa's most populous nations is a major maize producer on the continent, second to South Africa (FAOSTAT, 2017). Over 75% of Nigeria's maize is consumed by humans, as maize

is a staple of the Nigerian diet (USDA, 2014). With urbanization, higher incomes and increased animal protein consumption, Nigeria's demand for maize for feed has also been increasing rapidly. Between 2003 and 2015, the volume of maize used for feed in Nigeria increased from 300,000 to 1.8 million tons: a 600% increase (Liverpool-Tasie *et al*., 2017).

Despite the health risks associated with co-occurrence of aflatoxins and fumonisins in diets, few studies have explored the co-occurrence of these mycotoxins in foods consumed as key staples, and no such studies exist along supply chains in sub-Saharan Africa. Most studies on mycotoxins explore their prevalence (and/or strategies to reduce them) at particular nodes (e.g., on farms or in food). Very few consider how the structure of commodity supply chains and their interconnectedness to other commodity value chains during conversion to food and feed could affect mycotoxin prevalence. This is important because the maize value chain in Nigeria (as in many parts of Africa) is often a long and fragmented supply chain with many players involved (Liverpool-Tasie *et al*., 2017).

Since several previous studies demonstrated how both of these mycotoxins, alone and in concomitance are real concerns in toxicology, the aim of this study was to determine the extent of occurrence and cooccurrence of aflatoxins and fumonisins in the supply chain of Nigerian maize and maize-based products for both human consumption and animal feed.

2. Materials and methods

2.1 Study area

In this study, the occurrence and co-occurrence of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and fumonisins (FB_1, FB_2 and FB_3) along the maize value chain in southwest Nigeria is reported. Rather than just focusing on maize samples from one node of the value chain (e.g., maize from farmers or maize based products in retail outlets), we explore this phenomenon in samples

collected from actors all along the maize supply chain. This includes farmers and maize traders (after different lengths of storage), feed millers (maize and final feed) and retailers of maize based products.

The study area is Oyo State in Southwest Nigeria (Figure 2). Oyo State covers over 28,000 square kilometers with geographic coordinates 8°00′N 4°00′E. We selected this area (See Fig. 2) for several reasons. First, in addition to maize consumption by humans, southwest Nigeria (and Oyo State particularly) is a major zone for poultry production and aquaculture (USDA, 2018; Miller *et al*., 2006). Thus, this zone of the country is a major driver of increased maize demand (for animal feed) in the country. Second, the study area has a higher probability of human exposure to dietary mycotoxins. The majority of the maize in Nigeria is produced in the north, and then is moved over the country: often over a thousand kilometers to the south. Having to transport maize over such long distances creates potential additional opportunities for exposure to various molds. In addition to being a major consumption zone, the study area reflects the maize producing area of southwest Nigeria. Due to the very humid conditions in the southwest, the maize produced there is likely to face more challenges associated with exposure to moisture compared to the drier north. Though the study area is not nationally representative, it is largely representative of maize consumption and production areas in southwest Nigeria. Study samples were collected from farmers, traders, feed millers and retailers with appropriate institutional review board protocol.

2.2. Sampling of maize and maize products

Within the state, supply chain segments were selected based on their role within the maize poultry value chain. Thus, the specific local government areas for each node reflect the major source of the maize based product in the state. More details are provided for each node in the subsections below.

Farmer's sample. Farmers from two local government areas (LGAs: the third level of government administration in Nigeria, similar to counties in the USA) of Oyo State, Atisbo and Saki West, were selected for the samples of maize (Table 1). These two LGAs are the major maize producing LGAs in the state according to the Ministry of Agriculture. In each LGA, maize cobs were collected from 30 randomly selected farmers from the four main maize producing villages. For each farmer, 20 maize cobs were randomly selected from the farmer's field and store. Where available, unharvested maize cobs were randomly selected on farmer's field. Samples of maize cobs stored for minimum of one and maximum of four months were collected from each of the farmer's stores, where available. The samples were collected in two batches; first in January, 2018 then in March, 2018. At least two samples (from different points in time) were collected from each farmer giving 71 maize samples with 0–4 months of storage (see Table 1). The maize grain from the 20 cobs was shelled, hand-mixed and 500 g of grain were taken from each lot as a separate sample. 500 g of each maize grain were grounded separately with a milling machine and subsamples of 50 g were further taken from the lots and placed in a well-sealed and labeled polythene bag for mycotoxin analysis. Samples were stored at 4 °C prior to analyses.

Market samples. Three major maize wholesale markets in the Greater Ibadan area of Oyo State, Nigeria were selected for collection of maize samples from traders. One wholesale market is located in an urban area (Bodija market), one in a rural-near-city area (Ojaoba market) and the other in an off-market area (adjacent to but outside the actual market). Fifteen maize wholesalers were randomly selected from the three markets; five in each market. Samples consisting of 500 g maize grain were purchased from the sellers. The maize grains were ground separately with a milling machine and subsamples of 50 g were further taken from the lots and placed in a well-

sealed and labeled polythene bag for mycotoxin analysis. Samples were stored at 4oc prior to analyses.

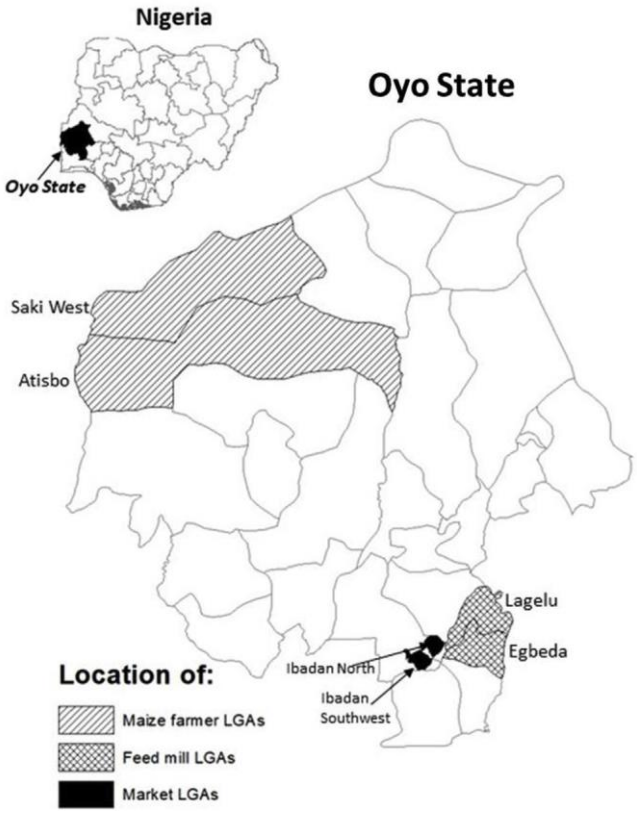
Table 1: De-identified farmer maize samples and duration of maize storage in major maize producing local government areas.

Local government	Serial number of farmers	Number of maize samples	Samples collected
Saki West	1	2	Stored
	2	2	Stored
	3	4	Stored
	4	2	Stored
	5	2	Stored
	6	4	Field/stored
	7	4	Field/stored
	8	2	Stored
	9	4	Field/stored
	10	2	Stored
	11	2	Stored
	12	2	Stored
Atisbo	1	2	Stored
	2	4	Field/stored
	3	4	Field/stored
	4	2	Stored
	5	4	Field/stored
	6	4	Field/stored
	7	2	Stored
	8	2	Stored
	9	2	Stored
	10	2	Stored
	11	2	Stored
	12	4	Field/stored
	13	1	Stored
	14	1	Stored
	15	1	Stored
	16	1	Stored
	17	1	Stored
Total		**71**	

Feed mill samples. Ten feed-mills from two LGAs (Lagelu and Egbeda) of the greater Ibadan area of Oyo state (identified by stakeholders in the poultry subsector as the areas with high concentrations of feed mills) were selected for the collection of poultry feed and maize samples. Five feed mills were randomly selected from a list of feed mills in each LGA and a sample of 500 g of finished feed and maize grain from the batch of maize used for producing the feed was collected from the feed-mills. Majority of these feed mills (90%) purchased their maize from the main maize producing regions of the state or the wholesale markets. The maize and feed samples from each feed miller were treated as separate samples linked to the same feed mill. A total of 10 maize grain and 10 poultry feed samples was collected from the feed mills. The maize grains were grounded separately with a milling machine and subsamples of 50 g were taken from each lot and placed in a well labeled polythene bag for mycotoxin analysis. The poultry feed was also labeled separately in polythene bag. Samples were stored at 4 °C prior to analyses.

Maize based processed products. Processed maize based products were purchased from the two main wholesales markets (Bodija and Ojaoba) in the study area. The identified products were broadly categorized into branded and unbranded maize based products. The branded products include cereals such as corn flakes, golden morn, and custard; while the unbranded products were largely maize based snacks sold informally called Kokoro and Aadun. A total of 44 processed maize products (34 branded and 10 unbranded) were purchased. They were well labeled and stored appropriately for mycotoxin analysis.

Figure 2: Map of study locations.



2.3. Mycotoxin analysis of maize samples

The maize samples were analyzed at Romer' lab (USA) using liquid chromatography tandem mass spectrometry (LC-MS/MS) for AFB1, AFB2, AFG1, AFG2, FB1, FB2 and FB3. The extraction of mycotoxins from the maize samples was carried out according to the method described by Sulyok *et al., (*2007). For each sample, 5 g were weighed and extracted with 20 ml of the extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v). For spiking experiments, 20 µl for AFB1, AFB2, AFG1, AFG2 and 50 µl for FB1, FB2 and FB3 of the combined working solutions were consecutively added to 0.25 g of each samples. The spiked sample was stored overnight at ambient temperature to allow evaporation of the solvent and to establish equilibrium between the analytes and the sample. Samples were extracted for 90 min on a GFL 3017 rotary shaker followed by

filtration. The filtered sample extract was diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v). The samples (except FB1, FB2 and FB3) were extracted, pushed through a Romer 228 MycoSep clean-up column, dried down, and reconstituted in internal standard out of which 40 µl were injected into the LC-MS/MS instrument. The fumonisin samples did not undergo any clean-up step. Apparent recoveries of the analytes were crosschecked by spiking a sample (multi-analyte standard on a fixed concentration level with no mycotoxin contamination). The corresponding peak areas of the spiked samples were then used to determine the apparent recoveries by comparison to a standard prepared and diluted in neat solvent. The concentrations of samples contaminated with aflatoxins and fumonisins were corrected by a factor equivalent to the reciprocal of apparent recovery (1/R; where R is the apparent recovery value) for each analyte.

LC-MS/MS parameters. The samples were screened for aflatoxin and fumonisin contamination using a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V electrospray ionization (ESI) source and a 1290 Series UHPLC System (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed on a Gemini R _ C18-column, 150mm×4.6 mmi d., 5 µm particle size, equipped with a C18 security guard cartridge, 4mm×3 mmi. d. (all from Phenomenex, Torrance, CA, USA) at room temperature. The analysis for all the mycotoxins were done in positive ion mode. For mobile phase A, DI H2O/formic acid with 1.2612 g ammonium formate was used as a solvent. Acetonitrile was used as the solvent for mobile phase B. Mycotoxin analyte identifications were confirmed by the acquisition of two MS/MS transition yielding 4 identification points. These are AFB1 parent ion: 313.1 m/z; product ions: 241.1 m/z and 285.0 m/z, AFB2 parent ion: 315.2 m/z; product ions: 287.0 m/z and 259.0 m/z, AFG2 parent ion: 329.1 m/z; product ions: 243.1 m/z and 115.1 m/z,

AFG2 parent ion: 331.1 m/z, product ions: 313.0 m/z and 115.1 m/z, FB1 parent ion: 722.4 m/z; product ions: 334.4 m/z and 352.4 m/z, FB2 parent ion: 706.4 m/z; product ions: 336.4 m/z and 318.4 m/z, FB3 parent ion: 706.3 m/z; product ions: 336.4 m/z and 318.5 m/z.

2.4 Data Analysis

Samples for which the aflatoxin and fumonisin levels were less than the limit of detection (LOD), the values were replaced with half of the limit of detection (LOD). All statistical analysis was done using MS Excel and the JMP 14 for Windows software. Kruskal–Wallis tests were performed to test the statistical significance for total aflatoxin and fumonisin levels among samples collected from farmers at different storage times. A Mann–Whitney test was used to compare the difference between two groups. A $P < 0.05$ was considered to be statistically significant for all the statistical tests.

3. Results

3.1. Farmers' samples

Table 2 shows how aflatoxin and fumonisin levels change over time in farmers stored maize grain, from harvest through to four months and more of storage.

Table 2: Geometric mean levels of each of the aflatoxins and fumonisins in farmers' maize samples, from harvest to four months and more in storage.

Months in storage	Number of samples	AFB_1 (µg/kg)	AFB_2 (µg/kg)	AFG_1 (µg/kg)	AFG_2 (µg/kg)	FB_1 (µg/kg)	FB_2 (µg/kg)	FB_3 (µg/kg)
Harvest (0)	8	1.40	0.60	1.12	0.80	765	562	191
1	10	2.28	0.73	0.80	0.80	462	175.0	76.3
2	19	4.27	0.79	1.10	0.93	390	190	78.9

Table 2 (cont'd)

3	24	12.2	1.25	1.04	0.83	689	223.0	93.7
4	8	27.9	3.27	2.67	1.35	745	299	96.5

As these results show, while levels of each of the aflatoxins generally increased with increasing amounts of time in storage, levels of each of the fumonisins generally decreased over time. Furthermore, while fumonisin stayed at levels generally considered safe during the duration of storage time measured, the same cannot be said for total aflatoxins. Aflatoxin levels at four months or longer in storage were exceedingly high: about 250 μg/kg, over all acceptable limits set for human food by nations worldwide (FAO, 2004).

Table 3 shows the total aflatoxin and fumonisin levels in maize samples collected from farmers, from harvest to 4 months of storage with 1-month intervals. The total aflatoxin level (AFB1 + AFB2 + AFG1 + AFG2) in the samples tends to increase with time of storage. The geometric mean of total aflatoxin level at harvest was 4.2 μg/kg, but after 4 months of storage, the level went up to 42.7 μg/kg: much higher than the Nigerian maximum total aflatoxin regulatory limit in maize of 4 μg/kg. At harvest, 37.5% of the samples had aflatoxin levels more than 4 μg/kg and after 4 months of storage 87.5% of the samples had aflatoxin levels exceeding 4 μg/kg. The geometric mean levels of total aflatoxin in the samples at different storage times were statistically significantly different ($p < 0.05$) (Table 6); higher aflatoxin levels with higher storage time. Notably, at the higher end of ranges in maize stored for four months or longer, aflatoxin levels were found to be so high as to be dangerous in causing acute toxicity in humans or animals.

However, the total fumonisin levels (FB1 + FB2 + FB3) do not follow any specific pattern with length of storage time. The highest geometric mean level of total fumonisin was observed in samples collected at harvest (1682 μg/kg); 37.5% of the samples collected at harvest had total

fumonisin levels higher than the United States Food and Drug Administration (USFDA) regulatory limit of 2000 µg/kg (USFDA, 2000). The geometric means of total fumonisin level across the groups were not significantly different ($p > 0.05$) (Table 7).

Table 3: Total aflatoxin and fumonisin levels (geometric mean and range) in maize stored for various lengths of time in farmers' households, Nigeria.

Months in storage	Mean total aflatoxin (µg/kg)	Range (µg/kg)	%>4 µg/kg aflatoxin	Mean total fumonisin (µg/kg)	Range (µg/kg)	%>2000 µg/kg fumonisin
Harvest (0)	4.2	2.7 – 26.5	37.5	1680	650 - 5800	37.5
1	5.3	2.7 - 42.5	50.0	671	200 - 3000	20.0
2	8.8	2.7 - 414	63.2	747	150 - 2300	21.0
3	17.5	2.7 - 180	91.7	1050	150 - 5800	20.8
4	42.7	2.7 - 1460	87.5	1230	650 - 2500	25.0

3.2. Maize from local maize traders

Table 4 panel A shows the total aflatoxin (AFB1 + AFB2 + AFG1 + AFG2) and fumonisin (FB1 + FB2 + FB3) levels in maize samples collected from maize traders after 1 week and 2 weeks of storage. The geometric mean of total aflatoxin level in maize stored for 1 week was only 3.0 µg/kg but after 2 weeks of storage, the level went up to 5.6 µg/kg. However, the geometric mean levels of total aflatoxin in the maize trader's samples at different storage times were not statistically significantly different ($p > 0.05$) (Table 6). The geometric mean level of total fumonisin in samples collected at 1 week was 665 µg/kg and 677 µg/kg at 2 weeks which were both lower than the European Union (EU) regulatory limit of 1000 µg/kg and according to Mann-Whitney U test, the geometric means of total fumonisin level cross the groups were not significantly different ($p > 0.05$) (Table 7).

3.3 Maize samples from feed millers

Table 4 panel B shows the total aflatoxin (AFB1 + AFB2 + AFG1 + AFG2) and fumonisin (FB1 + FB2 + FB3) levels in maize flour samples collected from feed millers from their storage and feed samples produced out of their stored maize. The geometric mean total aflatoxin level in the final feed (59.7 μg/kg) is much greater than that in the stored maize samples (3.1 μg/kg) and is statistically significantly different at $p < 0.05$ (Table 6). The geometric mean of total fumonisin level in the stored maize was 1040 μg/kg and 1330 μg/kg in the final feed, but the difference is not statistically significantly different ($p > 0.05$; Table 7).

Table 4: Aflatoxin and fumonisin levels (geometric mean and range) in maize flour samples collected from maize traders and poultry feed millers.

Maize flour storage time	No. of samples	Mean total aflatoxin (μg/kg)	Range (μg/kg)	Mean total fumonisin (μg/kg)	Range (μg/kg)
Maize traders (Panel A)					
1 week	9	3.0	2.7 – 7.9	665	350 - 900
2 weeks	5	5.6	2.7 – 54.9	677	150 - 2100
Feed millers (Panel B)					
Maize in storage	10	3.1	2.7 – 6.8	1410	850 - 4400
Final feed	10	59.7	20.3 - 297	819	150 - 4600

3.4. Branded and non-branded maize-based food products

Table 5 shows the total aflatoxin (AFB1 + AFB2 + AFG1 + AFG2) and fumonisin (FB1 + FB2 + FB3) levels in branded and non-branded snacks and cereals made from maize. The geometric mean total aflatoxin level in branded snacks-cereal mix and custard combined (2.9 μg/kg) is lower than that in the non-branded maize snack – corn roll (6.8 μg/kg). 4 out of the 34 (11.8%) branded snacks

and 8 out of the 10 (80%) non-branded snacks contained total aflatoxin levels higher than the Nigerian regulatory limits. The geometric mean of total aflatoxin levels between the branded and non-branded groups were significantly different ($P < 0.05$). The geometric mean total fumonisin level is also higher in the nonbranded snacks (335 μg/kg) compared to branded snacks (0–94 μg/kg). Though the mean levels in both groups were much lower than the US regulatory limits for fumonisins, the difference is statistically significant.

Table 5: Aflatoxin and fumonisin levels in branded vs non-branded snacks.

	Sample type	No. of samples	Mean total aflatoxin (μg/kg)	Range (μg/kg)	%>4 μg/kg aflatoxin	Mean total fumonisin (μg/kg)	Range
Non-branded	Corn roll	10	6.8	4.0 – 10.9	80.0	311	150 - 1050
Branded	Cereal mix	20	3.1	2.7 – 5.3	20.0	195	150 - 400
	Custard	14	2.7	2.7 – 2.7	0	150	150 - 150

As shown in Fig. 3, the geometric means of total aflatoxin levels in farmer's flour samples stored for 2–4 months, samples from maize traders stored for over 2 weeks, final feed samples from feed millers and the non-branded maize snacks were higher than 4 μg/kg which exceeded the Nigerian set maximum limit for total aflatoxin level in maize. The geometric means of total aflatoxin levels in other groups were comparatively lower and can be considered safe or acceptable. However, the geometric means of total fumonisin levels in all the group of samples collected were much less than the USFDA regulatory limit of 2000 μg/kg, as shown in Fig. 4.

Figure 3: Geometric means of total aflatoxin levels in Nigerian maize and maize products.

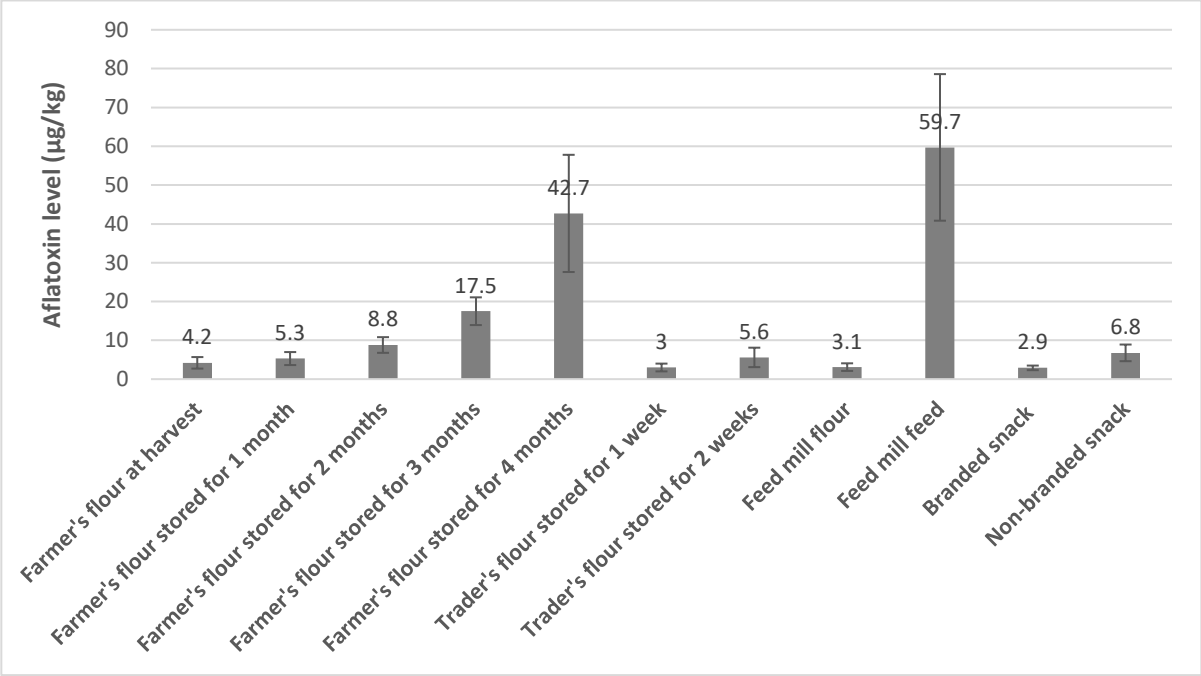


Figure 4: Geometric means of total fumonisin levels in Nigerian maize and maize products.

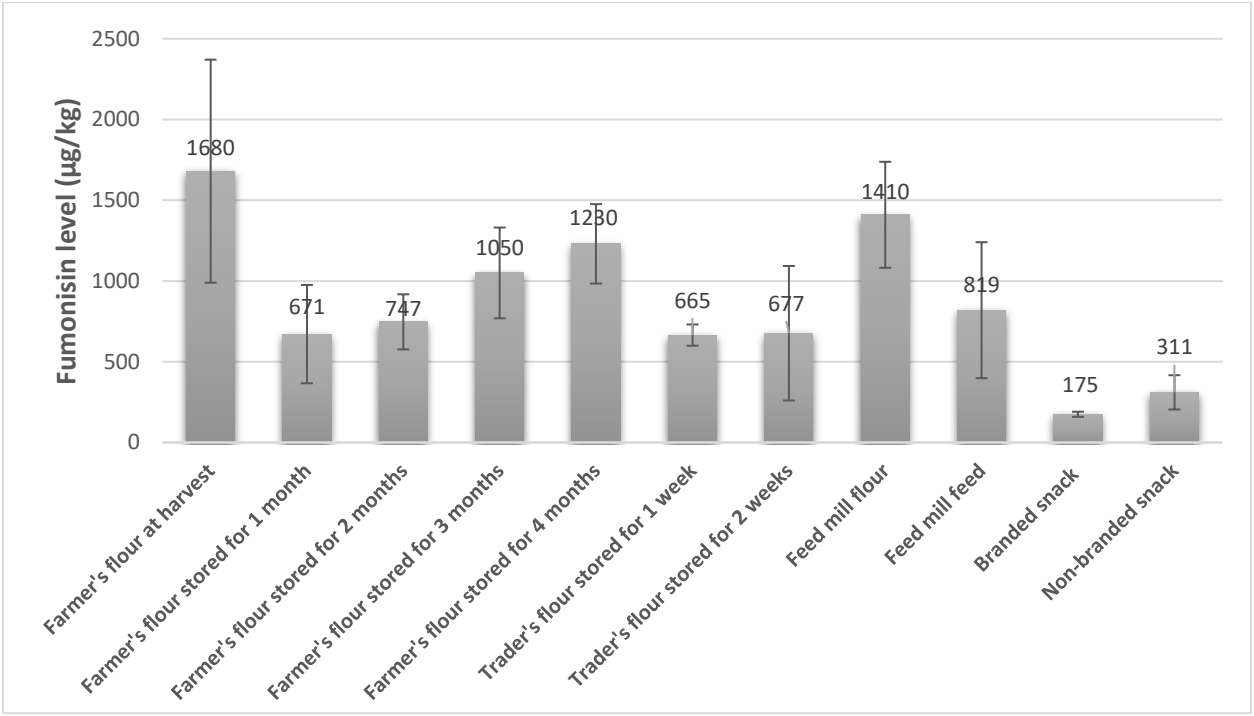


Table 6: Statistical analyses for aflatoxin levels across the groups.

Group	Statistical test used	P-value	U-value	Z-score
Farmer's flour (harvest to 4 months storage)	Kruskal-Wallis	0.00330*	-	-
Trader's flour (1 week to 2 weeks storage)	Mann-Whitney U	0.424	16	-0.8
Feed millers (stored maize to final feed)	Mann-Whitney U	0.000180*	0	-3.74
Branded and non-branded maize snacks	Mann-Whitney U	<0.0000100*	8	-4.52

*values significant with respect to a P-value of 0.05

Table 7: Statistical analyses for fumonisin levels across the groups.

Group	Statistical test used	P-value	U-value	Z-score
Farmer's flour (harvest to 4 months storage)	Kruskal-Wallis	0.125	-	-
Trader's flour (1 week to 2 weeks storage)	Mann-Whitney U	0.944	21.5	-0.0670
Feed millers (stored maize to final feed)	Mann-Whitney U	0.197	32.5	1.29
Branded and non-branded maize snacks	Mann-Whitney U	0.0128*	80.5	-2.49

*values significant with respect to a P-value of 0.05

4. Discussion

This work demonstrates the significant occurrence and co-occurrence of two important mycotoxins – aflatoxins and fumonisins – in Nigerian maize and maize products. This finding is important because maize is a staple food in Nigeria and many other sub-Saharan African nations, and these two toxins individually pose significant human health risks that may be increased by their co-occurrence in diets. Moreover, the co-occurrence is at multiple stages along the value chain of Nigerian maize: from harvest to postharvest storage to processing and final food and feed products in the marketplace.

The aflatoxin levels in samples collected from maize farmers indicate an increase in the aflatoxin levels with increasing time of storage. On average, total aflatoxins in farmer's samples stored for over 2 months–4 months exceeded the Nigerian regulatory limits for aflatoxins: 4 μg/kg, which is also considered unacceptable by European regulatory standards (EUC, 2006). There is no significant difference in mean levels of total fumonisins with the length of storage time, but almost 20.5% of the samples collected from the farmers and traders contained fumonisin levels higher than the US regulatory limits for fumonisin (2000 μg/kg). The mean of total aflatoxin level in the samples collected from maize traders that are stored for two weeks is greater than the mean of samples stored for one week. This finding supports previous studies that show aflatoxin levels increase with the time of storage in hot and humid countries as the combination of heat and dampness favors the growth of Aspergillus fungi, which produce aflatoxins (Villers, 2014). The total fumonisin levels in the samples collected both at one and two weeks of storage did not change as much.

The samples collected from feed millers demonstrate that even though mean levels of total aflatoxins in stored maize is low, the levels in the final feed are significantly higher. The drastic

increase in aflatoxins might be because other ingredients such as groundnut cake, which may also have aflatoxin contamination, are added to the feed. The total fumonisin levels were found to be lower in feed than in stored maize, and the geometric mean levels of total fumonisin in both the stored maize and final feed were much lower than the strictest US regulatory fumonisin level of 2 ppm in human food. The results from maize farmers and traders further confirm the potential for aflatoxin contamination during storage. This implies that efforts to reduce exposure to aflatoxins among maize consumers cannot only focus on one set of actors in the value chain. To focus only on maize production in the field is not likely to guarantee a safe product for the final maize consumer.

The feed mill results also reveal the interrelated nature of food supply chains. Issues of food and feed contamination require attention to be paid to related supply chains. Even though feed millers make efforts to secure a safe input (the mean aflatoxins levels in their maize were lower than the recommended levels), this does not guarantee a safe final feed product. Focusin exclusively on the maize supply chain does not necessarily guarantee improved safety of maize based products when combined with other ingredients, such as groundnuts in the case of feed.

In Nigerian branded and non-branded maize snacks, the geometric means of both total aflatoxin and total fumonisin levels tend to be much higher in the non-branded snacks than in branded snacks. Eighty percent of the non-branded snacks contained risky levels of total aflatoxins according to Nigerian and EU regulations. However, both the branded and non-branded snacks contained safe or allowable levels of total fumonisins, if compared to USFDA regulatory limits.

This study confirms that aflatoxins and fumonisins are prevalent contaminants of maize for human consumption and animal feed in Nigeria. A significant fraction (52%, 76 out of 147 samples collected) of maize and maize products was contaminated with aflatoxin levels above the Nigerian

maximum tolerable limit. In terms of fumonisins, 13% (19 out of 147 samples) of the total samples collected contained levels higher than the US regulatory limit of 2000 μg/kg. Regular routine checks by the Directorate of Food Safety and Nutrition (the directorate of the National Agency for food and drug administration agency mandated for such oversight) is still needed for the proper enforcement of existing standards. There is also a need for more oversight on fumonisins. This includes setting and enforcing standards on appropriate fumonisin levels.

Feasible and cost-effective methods to reduce aflatoxin risk in preharvest, postharvest, dietary, and clinical settings have been developed (Khlangwiset and Wu, 2010). Research and policy interventions that support the development and dissemination of improved maize varieties that are resistant to fungal infection and mycotoxin control on maize fields are important (Dorner and Horn, 2007). The International Institute of Tropical Agriculture (IITA) in Nigeria, along with other institutions worldwide such as the US Department of Agriculture, have worked on – among other strategies – developing aflatoxin-resistant maize hybrids with demonstrated efficacy in field conditions; although to date, none of these strains have been marketed (Brown *et al*., 2013). The absence of a price premium to compensate for investing in such technologies (e.g., AflaSafe, a biocontrol developed by IITA) limits their adoption in Nigeria (Ayedun *et al*., 2017). However, using such technologies alone is not enough to guarantee a safe maize product. In the absence of proper storage and handling practices or without taking into account the mycotoxin levels of other commodities mixed with maize in the production of final feed or food products, aflatoxin and fumonisin are likely to remain food safety challenges in maize-based products. Thus, these efforts may need to be accompanied by measures to prevent the exposure of grain to the fungi along the entire value chain, from harvest to food products in stores and homes. Due to the prevalence of multiple ingredients in most food and feed, minimizing human and animal exposure to dangerous

mycotoxins requires consideration of multiple related supply chains such as maize and groundnut products in the case of animal feed. Efforts to understand and address challenges associated with mycotoxins in maize-based products need to be more holistic and to consider the potential for exposure of the grain to these harmful fungi along the entire supply chain and across related supply chains.

CHAPTER THREE: Mycotoxin reduction through lactic acid fermentation: Evidence from commercial ogi processors in southwest Nigeria

This chapter has been previously published as Ademola, O., Saha Turna, N., Liverpool-Tasie, L. S. O., Obadina, A., & Wu, F. (2021). Mycotoxin reduction through lactic acid fermentation: Evidence from commercial ogi processors in southwest Nigeria. *Food Control*, 121, 107620.

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Contribution statement: Data Curation and Analysis, Writing – Original Draft Preparation.

Abstract

This work demonstrates the feasibility of a traditional food processing method to reduce mycotoxins (toxins produced by foodborne molds) in commercial processing plants in Nigeria. Aflatoxin, a commonly occurring mycotoxin in maize and nuts, causes liver cancer in humans, and has also been implicated in child growth impairment and immunotoxicity. Although fumonisin, another mycotoxin in maize, has not been conclusively linked to any human diseases, it causes multiple adverse effects in other animal species and may play a contributory role in neural tube defects and growth impairment in human children. This study examined the impact of lactic acid fermentation, a food processing method used for millennia across multiple human populations, to decrease aflatoxins and fumonisins in maize products in Nigeria. We assessed the prevalence of four aflatoxins and three fumonisins in matched samples of maize grain and a Nigerian porridge ogi (before and after processing) obtained from commercial ogi processors in three southwestern Nigerian states. After processing, the mean total aflatoxin level in the final product was typically close to the maximum acceptable limit shared by Nigeria and the European Union: 4 μg/kg. Lactic acid fermentation significantly reduced fumonisin levels in maize. As ogi is a common weaning food for Nigerian children, the fermentation process used to produce it is potentially beneficial in reducing mycotoxin-related health risks in a sensitive population. It is encouraging to see that

mycotoxin reductions occur even in commercial ogi production settings. However, the ultimate fate of these toxins warrants further investigation before this can be recommended as a public health intervention.

Key Words: Aflatoxin, Fumonisin, Lactic acid fermentation, Maize, Ogi, Nigeria

1. Introduction

Mycotoxins are toxins produced by fungi that colonize food crops and cause multiple adverse health effects, including cancer, in humans and animals. Aflatoxins and fumonisins are two major groups of foodborne mycotoxins of major concern in developing countries. Certain fungi of the genera *Aspergillus* and *Fusarium* that infect food crops, including maize, produce these particular toxins. They are of particular concern in maize in tropical and subtropical world regions, because warm climates encourage the growth of these fungi (Wu & Mitchell, 2016). First introduced to the African continent in the 1500s, maize has become a staple food crop throughout Africa. It accounts for 30–50% of low-income household expenditures in East and Southern Africa (IITA, 2013). It is also an important crop in West Africa, with Nigeria being among the two largest maize producing nations on the continent (FAOSTAT, 2017). While maize serves as an important ingredient for a rapidly growing animal feed industry in the country, humans consume 78% of the crop cultivated in Nigeria (USDA, 2017). Thus, the mycotoxins that naturally occur in maize are a concern for Nigerian public health.

All across Africa, maize is consumed in many different forms; including on the cob (boiled or roasted), wet or dry cereal, steamed custard, pudding, porridge, and maize gruel. A popular cereal produced from maize through fermentation in Nigeria is ogi. It is an affordable maize-based product consumed widely across the nation for breakfast. Ogi contains many nutritional benefits such as, minerals, vitamins, probiotics and high calories (Opere *et al*., 2012) and is easy to prepare

which makes it preferable by almost 150 million people living in West Africa (Oguntoyinbo & Narbad, 2012). Ogi is also a very important weaning food for infants and a convenient meal for young children and those convalescing from illness (Onyekwere *et al*., 1989). Because of the consumption of ogi by potentially vulnerable populations such as young children and the elderly or ill, it is important to consider the risk of mycotoxins in this food product.

Aflatoxin and fumonisin are two of the most prominent mycotoxins in maize and maize products. Aflatoxins have been estimated to cause 25,000–155,000 liver cancer cases worldwide per year, and to make up nearly a quarter of all liver cancer cases in high-exposure world regions including Africa (Liu *et al*., 2012; Liu & Wu, 2010); while fumonisins have been associated with neural tube defects in infants whose mothers were exposed during pregnancy (Missmer *et al*., 2005). In the past, fumonisin exposure was also associated with increased risk of esophageal cancer, although the evidence is more limited (Rheeder *et al*., 1992). There is also increasing evidence that exposure to mycotoxins may compromise immunity and contribute to stunted growth in children (Chen, Mitchell, *et al*., 2018; Chen, Riley, *et al*., 2018; Gong *et al*., 2004; Jiang *et al*., 2005; Khlangwiset *et al*., 2011; Mahdavi *et al*., 2010; Shuaib *et al*., 2010; Turner *et al*., 2007; Wu *et al*., 2014). Consequently, mycotoxin reduction in commodities such as ogi frequently consumed by households and children should be a food safety priority.

Many common methods of food processing may reduce mycotoxin levels. Physical, chemical, enzymatic and microbial methods of food processing that have been shown to decrease mycotoxin levels include sieve cleaning, flotation density sorting, baking, frying, roasting, sorting, milling and extrusion (Karlovsky *et al*., 2016; Kaushik, 2015; Voss *et al*., 2017). Ogi production includes a natural process of fermentation caused by the presence of microorganism in the environment. Previous studies analyzing the microbial diversity in ogi production have indicated LAB to be the

dominant species in the fermentation process (Oguntoyinbo *et al*., 2011; Oguntoyibo and Narbad, 2012; Omemu, 2011; Oyedeji *et al*., 2013). Processing through lactic acid fermentation has been shown in numerous studies to significantly reduce levels of mycotoxins including aflatoxins and fumonisins (Chilaka *et al*., 2019; Khlangwiset & Wu, 2010; Mokoena *et al*., 2006; Nyamete *et al*., 2016; Okeke *et al*., 2015, 2018; Roger *et al*., 2015; Shetty & Jespersen, 2006; Zhao *et al*., 2015). Whether these reductions have been accompanied by improved health benefits is uncertain, however, currently, there is limited rigorous analysis of this phenomenon – lactic acid fermentation reducing mycotoxins - in foods processed in Nigeria. Adegoke *et al., (*1994), and Okeke *et al., (*2015) and Adegoke *et al., (*1994) did not examine changes in fumonisin levels, but focused on just one aflatoxin, AFB1 (the most toxic of the aflatoxins). Furthermore, Adegoke *et al., (*1994) used the thin layer chromatography method (TLC), while quantified mycotoxin levels with the enzyme linked immunosorbent assay (ELISA). However, due to the complexity of analyzing food samples coupled with possible low concentrations at which mycotoxin contamination can occur, a highly sensitive, selective, and reliable analytical method for mycotoxin quantification is required. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a more recent methodology that meets these requirements, and was used in this study to quantify the levels of seven mycotoxins including the four common aflatoxins (AFB1, AFB2, AFG1, and AFG2) reported to be present in agricultural produce. This study also considers the three fumonisins frequently reported in food: FB1, FB2, and FB3. Okeke *et al.*, (2015) found that steeping maize for 48 h or longer could significantly reduce multiple mycotoxins, and that fermentation of maize to ogi could significantly reduce cyclopiazonic acid and aflatoxin M1 (AFB1 levels were already low, and percentage reduction could not be determined). It is the only study in Nigeria where the authors have used LC-MS/MS to explore the effect of lactic acid fermentation on mycotoxins reduction in

Nigeria. However, that study was restricted to one location, and the study explored the effects of processing on mycotoxins for laboratory-processed ogi. Since consumers usually purchase ogi in wet form from the processors, studying commercially processed ogi is important to understand how safe this commercially produced food product is, and how the levels and potential reduction of aflatoxins and fumonisins vary with processing practices. For example, higher levels of mycotoxin exposure occur when moldy, broken and damaged maize grains are used (Ediage *et al*., 2013; Ezekiel *et al*., 2014). The quality of the raw material used actually influences the safety of fermented food products (Steinkraus, 1983). Studies have also shown that processing practices (Sadiku, 2010), the processing environment and hygiene of the personnel performing the art of fermentation (Iwuoha & Eke, 1996) are also key determinants of the safety of fermented products.

Thus far, studies have examined how lactic acid fermentation affects mycotoxin levels in laboratory-fermented ogi. To gain a more real-to-life understanding of the impacts of lactic acid fermentation on maize in our study, we have analyzed the mycotoxin levels in commercially produced ogi (fermentation done by ogi producers themselves) sold in Nigeria. This study helps to fill that gap. In this study, we assessed the prevalence of four aflatoxins and three fumonisins in matched samples of maize grain and ogi (before and after processing the original maize) collected from commercial ogi processors in southwestern Nigeria. The study determines the extent to which lactic acid fermentation reduces mycotoxin levels in this important staple food in Nigeria.

2. Materials and methods

2.1. Study area

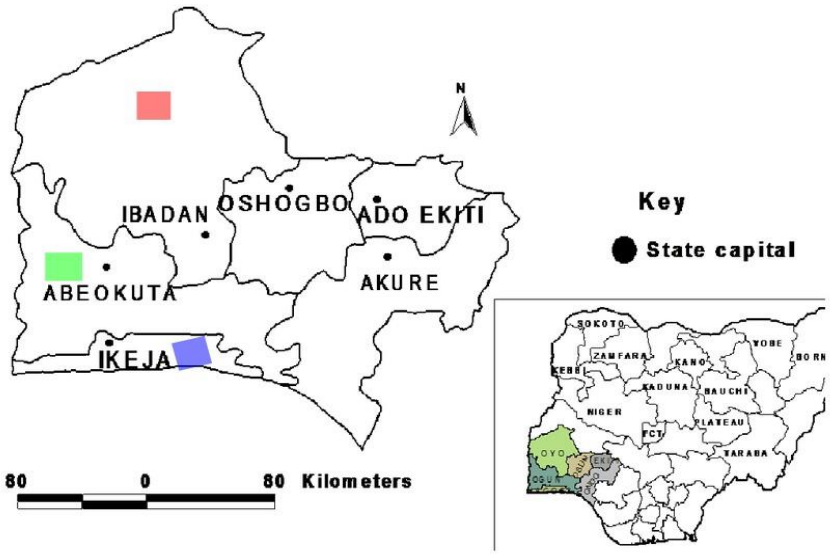
For this study, we sourced maize and ogi from ogi processors in three southwestern states in Nigeria (Fig. 2). This region of the country was selected because of its high maize demand for both human food and animal feed. Moreover, the area largely depends on maize from northern Nigeria,

where the majority of the nation's maize is produced. Thus, the relatively long supply chain for maize to reach the southwest could render the region more susceptible to mycotoxin contamination. Three towns - Ibadan, Abeokuta, and Ikeja - were selected (one in each state), due to the presence of dense ogi commercial centers.

2.2 Sources of maize grain and ogi

We collected maize grain (raw material) and ogi produced from the same maize grain (fermented maize final processed product) from ten randomly selected ogi processors in each of the three study locations in April, 2018. While a formal listing was not conducted in the commercial centers/markets, ogi processors were systematically selected across the different parts of the markets and times of operation within a day. To understand the factors that could affect how mycotoxin levels and their relative reduction varied with processor practices, we administered a structured questionnaire to each processor about their maize storage and processing practices (Appendix 1), as warm and damp storage conditions can increase aflatoxin accumulation (Bradford *et al*., 2018). We collected the maize grain (raw material) samples first and went back to collect the ogi (final product) samples after the processing was done at each study location. The ogi samples were matched to the original maize from which they were produced. Five hundred grams (500 g) of maize grain were collected from each ogi processor. Fifty grams (50 g) from each milled sample were packed in aseptic polythene bags for mycotoxin analyses. Fifty grams (50 g) of the final product (ogi) were also purchased from each processor. The ogi was packed and labeled in a similar manner as the maize grain, transferred to the laboratory aseptically and both were stored at - 20 ◦C prior to mycotoxin analysis. Sixty samples (30 maize and 30 ogi) were obtained in total from all of the processors.

Figure 5: Map of southwest Nigeria indicating the study locations.



Note: The top left map shows the three study locations within their respective Nigerian states. Ikeja is the study location in Lagos State (purple box) while Abeokuta is the study location in Ogun State (green box) and Ibadan is the study location in Oyo State (red box) The bottom right map highlights the Nigerian states where the study locations (in the top left) are found. Oyo is light green; Ogun state is dark green and Lagos is depicted in brown. Source: www.researchgate.net/figure/map-of-southwest-Nigeria-showing-capital-citiesinset-map-of Nigeria_fig1_228532647/amp.

2.3. Commercial versus laboratory processing method of ogi

The general processing procedure for ogi production was similar across the three study locations. Maize grains were soaked in water and allowed to ferment (steeping) for 2–4 days (48–96 h). The softened grains were then washed, wet milled, and sieved using a muslin cloth. The sieved paste was diluted with water in a container and left to ferment (souring) for 1–2 days (24–48 h). The surface water was decanted, and the sediment (wet paste) allowed to stand to solidify. The solidified product was then measured into small units in clear polythene bags for sale. To distinguish potential practices that might affect mycotoxin reduction through ogi production, the

practices of commercial processors were compared to the laboratory procedures described in Adebayo and Aderiye (2007). The main differences between the laboratory processing of ogi and commercial processing are that there was a sorting stage before steeping in the lab processing that is not done by commercial processors (Fig. 6); and the laboratory processing had no second fermentation step (souring), which ogi processing companies often employ.

2.4. Mycotoxin analysis of maize and ogi samples

2.4.1. Extraction of maize grains and ogi samples

The labeled maize and ogi samples were sent to Romer Labs, USA, for mycotoxin analyses. Mycotoxin analyses of maize grain and ogi samples were performed by using LC-MS/MS because of the low limit of detection of mycotoxins and multi-toxins it can determine. The extraction of maize and ogi samples, apparent recoveries of analytes, and mycotoxin analyses were performed according to the method described by Sulyok *et al., (*2007). For most of the samples, 25 g of each sample was weighed into a polypropylene tube and extracted with 100 ml of the extraction solvent (acetonitrile/DI water 84:16 (for aflatoxin) and 50:50 (for fumonisin), v/v by volume). For some samples, there was not enough material to weigh out 25 g. In those cases, either 12.5 g/50 ml or 5 g/20 ml extractions were done keeping the ratio of sample to extraction solvent constant at 1:4. For spiking experiments, samples were extracted for 90 min on a GFL 3017 rotary shaker and diluted with the same volume of dilution solvent (acetonitrile/DI water). 40 µl of the diluted extracts were injected into the LC instrument. Apparent recoveries of the analytes were cross-checked by spiking a sample that was not contaminated with mycotoxins with a multi-analyte standard on one concentration level. The spiked sample was stored overnight at ambient temperature to allow evaporation of the solvent and to establish equilibrium between the analytes and the sample. For quality control, a seven (7) point calibration curve containing the mycotoxins

prepared and diluted in neat solvent is also injected and analyzed with every LC-MS/MS batch run. The corresponding peak areas of the spiked samples were then used for the estimation of apparent recoveries by comparison to the standard. All concentrations of the naturally contaminated samples were corrected by a factor equivalent to the reciprocal of apparent recovery (1/R; where R is the apparent recovery value) of each analyte. Sample results were adjusted based on the recoveries that were obtained.

2.4.2. LC-MS/MS parameters

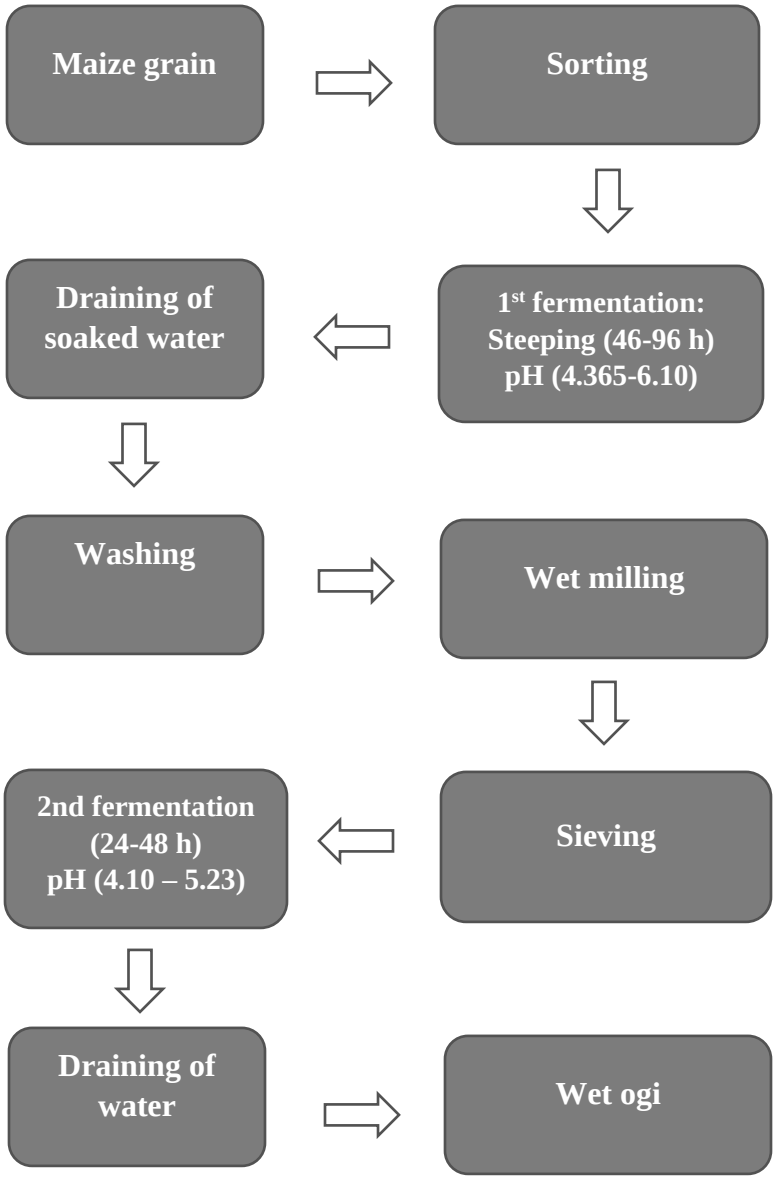
Mycotoxins (aflatoxins and fumonisins) were screened using a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V electrospray ionization (ESI) source and a 1290 Series UHPLC System (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 ◦C on a Gemini R _ C18-column, 150 mm × 4.6 mmi d., 5 μm particle size, equipped with a C18 security guard cartridge, 4 mm × 3 mmi. d. (all from Phenomenex, Torrance, CA, USA). Positive analyte identification was confirmed by the acquisition of two MS/MS transitions, which yielded 4.0 identification points according to commission decision 2002/657/EC.

2.5. Data analysis

Descriptive statistics were used to explore the occurrence and concentration of aflatoxins and fumonisins in maize and ogi obtained across the three study locations. The non-parametric Wilcoxon sign rank test of matched pairs was used to test for significant differences in mycotoxin levels before and after processing. Next, the study explored differences in mycotoxin levels based on processing practices, which take place before/during ogi processing. Processors were divided into groups depending on how long they steeped their maize during processing and how long they

stored their maize before processing. To test the effect of these practices on mycotoxin levels, the non-parametric two-sample Wilcoxon rank-sum (Mann-Whitney) test was used. A $P \leq 0.05$ was considered to be statistically significant for all the statistical tests.

Figure 6: Flow chart of commercial processing of ogi. Generated by authors based on steps followed by commercial processors in the study. This was compared to the lab procedure articulated in Adebayo and Aderiye (2007).



3. Results

3.1. Characteristics of the ogi processors

The procurement and storage practices of the study processors across the three locations are presented in Table 8. Seventy-three percent (73%) of the processors stored their maize for less than seven days while 27% stored maize for more than seven days. About 43% of the processors did not store their maize before processing; thus, reducing the risk of mycotoxin accumulation in storage. This is because they typically buy small quantities from the market; just enough to produce their desired quantity of ogi. For those who did store, the most common storage method used across the three locations was a plastic container; used by about 33% of processors. The plastic containers are made from hard plastic and typically uncovered. Thus, exposure to moisture and heat is likely to be high. Thirty-two percent (32%) and three percent used a jute bag and polythene bag respectively. The majority of the processors (90%) claimed not to have problems with insects/rats/mold infestation, and 67% reported cleaning their storage structures before use. During the process of ogi production, no processors sorted their maize before steeping (soaking the maize grain for initial fermentation). Forty percent (40%) steeped their maize for 2 days, fifty-seven (57%) for three days and three percent (3%, one sample) steeped for four days. While most processors in Ibadan and Abeokuta steeped the maize for two days, 70% of processors in Lagos steeped for three days. Most processors (97%) allowed their maize to undergo souring (soaking of the milled maize for additional fermentation) for one day while only one processor soured for 2 days.

Table 8: Storage and processing characteristics of the *ogi* processors.

Parameters	Number observed (%)
	Total
Length of storage	
< 7 days	22 (73)
>7 days	8 (27)
Storage structure	
Plastic container*	10(33.3)
Jute sack on cemented floor*	6 (20)
Polythene bag*	1 (3.3)
None	13 (43.3)
Location of purchase of maize	
South	30 (100)
North	None
Reported problem with insects/rats/mould	
Yes	3 (10)
No	27 (90)
Cleaning of storage structure before use	
Yes	10 (33)
No	20 (67)
Sorting of maize before processing	
Yes	None
No	30 (100)
Number of days of steeping/soaking	
2	12 (40)
3	17 (57)
4	1 (3)
Number of days of souring	
1	29 (97)
2	1 (3)

Note: * means conditional on storing

3.2. Occurrence of aflatoxins and fumonisins in maize grain and ogi, before and after processing

Seven mycotoxins - AFB1, AFB2, AFG1, AFG2, FB1, FB2, and FB3 - were quantified in all samples. The limit of detection (LOD) for aflatoxins ranged from 1.1 to 1.6 μg/kg, while the LOD for fumonisins was 100 μg/kg. The recovery data for AFB1, AFB2, AFG1 and AFG2 were 115.84%, 121.70%, 115.17% and 125.39% while FB1, FB2, and FB3 were 109.18%, 94.62% and 94.05% respectively. Maize samples obtained from Ibadan and Abeokuta tended to have higher levels of mycotoxins than Lagos. The geometric mean for total aflatoxin level in maize samples from Ibadan before fermentation was 8.21 μg/kg while the geometric mean for total aflatoxin level in the ogi samples (after fermentation) was 2.38 μg/kg. In Abeokuta, the geometric mean for total aflatoxin level in maize and ogi were 3.90 μg/kg and 3.20 μg/kg respectively. Maize samples from Lagos had geometric mean total aflatoxin consistently less than LOD. We reject the null hypothesis that our data for both the maize and ogi samples are normal. Thus, a non-parametric Wilcoxon sign rank test of matched pairs was used to compare the geometric means of aflatoxin levels before and after processing. For maize samples that had aflatoxin and fumonisin levels less than LOD, the values were replaced with quarter of LOD for each mycotoxin. We found that the levels of AFB1, AFG1, AFG2 and total aflatoxin after processing (in ogi) were significantly lower than the initial levels in the maize grain in Ibadan. In Abeokuta, the AFB1, AFB2 and total aflatoxin levels in ogi were lower than initial levels in the maize grain but the differences were not statistically significant. The geometric mean levels of the different aflatoxins studied were not statistically significantly different after processing in Lagos, possibly because initial levels of aflatoxin were not high in maize here.

For fumonisins, prior to ogi processing including fermentation, the geometric mean of total fumonisin in maize samples were 465.9, 142.7 and 192.6 μg/kg for Ibadan, Lagos and Abeokuta,

respectively. After processing, the geometric mean levels of total fumonisin in the fermented product (ogi) were 133.2, 75.0 (quarter of LOD) and 90.67 µg/ kg for Ibadan, Lagos and Abeokuta. The fumonisin levels in ogi were significantly lower than the levels in the raw material (maize grain) in all the cities, but only significantly lower in samples collected from Ibadan ($P < 0.05$) according to Mann-Whitney *U* test.

The percentage reduction of aflatoxins and fumonisins in maize due to processing, including lactic acid fermentation, across the three locations is shown in Table 9. Estimates were based on percentage differences between aflatoxin and fumonisin levels in the maize grain and final product (ogi). For AFB1, AFG1 and AFG2 in Ibadan, the percentage reduction levels were 82.63%, 81.88% and 0.0% respectively while for AFB2, the level increased by 15.79%. For AFB1, AFB2 and AFG2 in Abeokuta, the percentage reduction level was 10.78%, 37.45% and 0% respectively while for AFG1, the level increased by 11.46%. No significant reduction in aflatoxin in maize sourced from Lagos could be found, because the initial levels of aflatoxin were already below the analytical limit of detection (LOD). For FB1 and total fumonisins, high and significant levels of percentage reduction in maize grain from ogi processing was observed in Ibadan and Lagos. For FB1 the percentage reduction level in Ibadan and Lagos were 84.88% and 66.05% respectively. For total fumonisins, the percentage reduction levels were 71.40% and 47.45% for Ibadan and Lagos respectively. This confirms that ogi processing, including fermentation of maize influenced by lactic acid bacteria (LAB), is associated with significant reductions in fumonisins in southwest Nigeria1. This finding is consistent with Okeke *et al., (*2015), who reported approximately 85% reduction in fumonisins in white and yellow maize grain for ogi production in Ogun state Nigeria. However, it contrasts with the findings of Fandohan *et al., (*2005), who reported small (and statistically insignificant) effects of lactic acid fermentation on fumonisin levels (13%) in the

Republic of Benin. Though the findings of this study are consistent with those of Okeke *et al.*, *(*2015), the reduction levels for the different mycotoxins found in this study are consistently lower than theirs. This might be due to external factors and processing practices adopted by processors not accounted for in a laboratory setting and reflects the importance of conducting a study with actual processors. When samples collected from all three cities were combined, we found 38.1% reduction in total aflatoxin and 58.6% reduction in total fumonisin after processing (significant reduction only for total fumonisin levels; $P = 0.0001$).

Table 9: Percentage reduction of aflatoxin and fumonisin in fermented *ogi* due to fermentation of maize.

Location	Initial level of AFB_1 (µg/kg)	SE	(%) reduction of AFB_1	Initial level of AFB_2 (µg/kg)	SE	(%) reduction of AFB_2	Initial level of AFG_1 (µg/kg)	SE	(%) reduction of AFG_1	Initial level of AFG_2 (µg/kg)	SE	(%) reduction of AFG_2	Initial level of total aflatoxins (µg/kg)	SE	(%) reduction of total aflatoxin
Ibadan	5	1.04	82.63	0.3	0	-15.79	2.27	0.56	81.88	0.4	0	-	8.21	1.57	71.07
Lagos	0.325	0	-	0.3	0	-	0.275	0	-	0.4	0	-	1.3	0	-
Abeokuta	1.49	11.54	10.78	0.55	1.83	37.45	0.41	0.24	-11.46	0.4	0	-	3.9	13.33	18.04
All cities combined	1.34	3.89	46.29	0.37	0.61	10.2	0.64	0.29	41.32	0.4	0	-	3.47	4.51	38.1
Location	**Initial level of FB_1 (µg/kg)**	**SE**	**(%) reduction of FB_1**	**Initial level of FB_2 (µg/kg)**	**SE**	**(%) reduction of FB_2**	**Initial level of FB_3 (µg/kg)**	**SE**	**(%) reduction of FB_3**				**Initial level of total fumonisin (µg/kg)**	**SE**	**(%) reduction of total fumonisin**
Ibadan	379.77	102.47	84.88	45.33	30.61	10.4	28.72	7.5	12.94				465.88	136.33	71.4
Lagos	73.64	43.15	66.05	25	0	-	25	0	-				142.72	43.15	47.45
Abeokuta	95.88	92.05	63.12	45.33	30.61	44.85	25	0	-				192.6	121	52.92
All cities combined	138.9	52.55	73.35	37.17	14.66	20.94	26.18	2.5	4.52				243	66.78	58.64

Note: FBs refers to fumonisins, AFBs refers to aflatoxins
SE = standard error
All the levels are listed as geometric means

3.3. Effect of processing practices and storage on mycotoxin concentrations

3.3.1. The effect of length of steeping on the levels of aflatoxins and fumonisins

Steeping is an important process of maize grain fermentation prior to milling, because it releases bacteria which allows for the breakdown of protein matrix Karlovsky *et al., (*2016). The LAB genera that occur in maize steep liquor are *Lactobacillus* and *Leuconostoc*, reported by Oyedeji *et al., (*2013). Okeke *et al., (*2015) also determined the occurrence and dominance of *L. paraplantarum, P. acidilactici, P. claussenii* and *P. pentosaceus* at different steeping times of the maize. Water-soluble toxins (fumonisins) migrate from grains to steep water, which facilitates mycotoxin reduction (Canela *et al*., 1996). Steeping time among the study processors ranged between two and four days. We only had one sample that was steeped for four days; therefore, we did not include that sample for statistical significance calculations of mycotoxin levels at different steeping durations. Table 10 shows the geometric mean levels of aflatoxins and fumonisins in ogi at different steeping durations. Our results from the Wilcoxon rank sum (Man Whitney U) tests show that the levels of aflatoxins and fumonisins in ogi were not significantly different for those who steeped for the recommended number of days (two) and those who did not (in our data this would be steeping for three days). Across the three study locations, there is no statistically significant difference in the geometric mean levels of total aflatoxin due to the length of steeping in the three study locations. When samples from all three cities were combined, there was no significant difference observed between mycotoxin levels in samples steeped for two days and samples steeped for three days. Therefore, steeping the maize for longer than two days did not result in significant reduction of aflatoxin or fumonisin levels. The limited significant differences in geometric mean levels for these mycotoxins due to different lengths of steeping, might be due to the limited variation in the number of days of steeping in our sample and might indicate that the

general steeping practices of processors do not significantly affect the effectiveness of lactic acid fermentation. Previous studies have found that extended fermentation could increase acidic conditions to a level that would interfere with mycotoxin reduction and also may cause aflatoxin to reform (Kpodo *et al*., 1996; Okeke *et al*., 2015). Their results could also explain why, in the current study, the levels of mycotoxins went up at some instances and no significant mycotoxin reduction occurred when steeped for longer than two days.

Table 10: Geometric Means of aflatoxins and fumonisin level in *ogi* found at different steeping duration.

Location	N	Duration of steeping (days)	Levels of aflatoxins (µg/kg)				
			AFB_1	AFB_2	AFG_1	AFG_2	Total aflatoxins
Ibadan	2	2	7.01±3.95	0.30±0.0[b]	3.17±1.35	0.40±0.0[b]	10.93±5.30
	7	3	4.38±1.10	0.30±0.0[b]	1.93±0.74	0.40±0.0[b]	7.27±1.83
Lagos	7	2	0.33±0.0[b]	0.30±0.0[b]	0.28±0.0[b]	0.40±0.0[b]	1.30±0.0
	3	3	0.33±0.0[b]	0.30±0.0[b]	0.28±0.0[b]	0.40±0.0[b]	1.30±0.0
Abeokuta	3	2	0.33±0.0[b]	0.30±0.0[b]	0.28±0.0[b]	0.40±0.0[b]	1.30±0.0
	7	3	2.87±16.24	0.71±2.60	0.49±0.34	0.40±0.0[b]	6.25±18.74
All cities combined	12	2	0.54±0.99	0.30±0.0[b]	0.41±0.39	0.40±0.0[b]	1.86±1.39
	17	3	2.33±6.77	0.43±1.08	0.78±0.41	0.40±0.0[b]	5.04±7.80
Location	N	Duration of steeping (days)	Levels of fumonisins (µg/kg)				
			FB_1	FB_2	FB_3		Total fumonisins

Table 10 (cont'd)

Ibadan	2	2	509.9±550	86.60±137.50	37.50±53.03		651.9±725
	7	3	346.5±52.16	41.02±25.51	25.00±0.0[b]		425.3±67.42
Lagos	7	2	64.59±43.93	25.00±0.0[b]	25.00±0.0[b]		131.8±43.93
	3	3	100.0±114.56	25.00±0.0[b]	25.00±0.0[b]		171.7±114.56
Abeokuta	3	2	57.24±91.67	25.00±0.0[b]	25.00±0.0[b]		125.33±91.67
	7	3	119.60±124.85	58.50±41.55	25.00±0.0[b]		231.53±165.18
All cities combined	12	2	88.43±103.3	30.75±22.92	28.06±6.25		169.1±131.3
	17	3	179.6±58.55	43.5±20.36	25.00±0.0[b]		282.1±75.01

Note: * = significant difference between steeping durations two and three ($p<0.05$), there was no significant difference observed between mycotoxin levels at steeped samples (day 2 vs day 3)

n = number of observations

[a] indicates number of samples = 1

[b] indicates samples with levels below LOD; these values were replaced with quarter of the LODs for each mycotoxin

3.3.2. The effect of length of maize storage on the levels of aflatoxins and fumonisins

Table 11 shows the effect of length of maize grain storage on the levels of aflatoxins and fumonisins concentration before processing (lactic acid fermentation) across the three study locations in southwestern Nigeria. The length of maize storage in our sample ranged from 0 to 14 days and varied across locations. The average number of days that maize was stored by ogi processors was seven in Ibadan and Abeokuta while it was eight in Lagos. Processors were divided

into two groups based on how long they stored their maize grain before processing. The first group consisted of those who stored maize grain for fewer than seven days before processing, while the second group included those who stored for seven or more days. We only found significant difference in levels of AFG1, FB1 and FB2 in samples collected from Abeokuta, where mycotoxin levels were higher among processors who stored maize for more than six days. Nevertheless, the levels were still lower than the regulatory limits. There were no statistically significant differences observed when samples from all cities were combined in the two storage groups and then compared through the Mann-Whitney *U* test. The limited evidence of difference in mycotoxin levels between these two groups might be driven by the generally low storage periods of the maize (typically less than 2 weeks) and also small sample sizes (n = 2 to 3) for some of the comparisons.

Table 11: Geometric means of aflatoxin and fumonisin levels in maize at different storage durations.

Location	**N**	**Length of storage (days)**	**Geometric mean level of aflatoxin (µg/kg)**					**Geometric mean level of fumonisin (µg/kg)**			
			AFB_1	AFB_2	AFG_1	AFG_2	Total aflatoxins	FB_1	FB_2	FB_3	Total fumonisins
Ibadan	7	0-6	5.36±1.39	0.30±0.0[a]	2.32±0.76	0.40±0.0[a]	8.72±2.11	348.8±144.3	58.5±41.6	30.48±10.71	485.6±192.8
	3	7-14	4.23±1.32	0.30±0.0[a]	2.15±0.59	0.40±0.0[a]	7.13±1.91	368.4±100.0	25.0±0.0[a]	25.0±0.0[a]	422.9±100.0
Lagos	7	0-6	0.33±0.0[a]	0.30±0.0[a]	0.28±0.0[a]	0.40±0.0[a]	1.30±0.0[a]	71.3±59.7	25.0±0.0[a]	25.0±0.0[a]	143.4±59.69
	3	7-14	0.33±0.0[a]	0.30±0.0[a]	0.28±0.0[a]	0.40±0.0[a]	1.30±0.0[a]	79.4±50.7	25.0±0.0[a]	25.0±0.0[a]	141.2±50.69
Abeokuta	8	0-6	1.15±14.5	0.64±2.28	0.28±0.0*	0.40±0.0[a]	3.36±16.73	57.3±39.65*	29.7±9.38*	25.0±0.0[a]	127.0±43.81

Table 11 (cont'd)

	2	7-14	4.30 ±0.20	0.30± 0.0	2.10± 0.10*	0.40 ±0.0 [a]	7.09±0.3	748.3± 50.0*	245.0±50 .0*	25.0±0.0 [a]	1020±10 0.0
All cities combined	22	0-6	1.25 ±5.30	0.40± 0.84	0.54± 0.37	0.40 ±0.0	3.36±6.13	112.6± 60.69	34.90±14 .80	26.63±3. 41	202.2±76 .53
	8	7-14	1.62 ±0.88	0.30± 0.0	0.99± 0.41	0.40 ±0.0	3.76±1.28	247.3± 101.5	44.23±38 .02	25.0±0.0	349.3±13 3.8

Note: * = significant difference between storage durations 0-6 and 7-14 at ($p<0.05$) n = number of observations.

[a] indicates samples with levels below LOD; these values were replaced with quarter of the LODs for each mycotoxin

4. Discussion

Reductions in both aflatoxins and fumonisins were achieved by fermenting maize into ogi by lactic acid fermentation. In ogi samples sourced from Ibadan, these reductions were statistically significant. While aflatoxin levels in the processed ogi in Abeokuta were lower than in the original maize grain, these differences were not statistically significant. In Ibadan and Lagos, FB1 and total fumonisin levels were significantly lower after fermentation with LAB. This suggests that lactic acid fermentation is still able to significantly reduce the levels of these toxins.

The geometric mean and median levels of total fumonisins in all three study locations were generally below maximum acceptable limits of 1000 µg/kg set by the European Union for maize grain (EUC, 2006); currently, Nigeria does not have food safety regulations for fumonisin. Our results for aflatoxin reductions from lactic acid fermentation are consistent with Fandohan *et al., (*2005) and Okeke *et al., (*2015). However, these two studies did not find - as this current study does, consistently significant reductions in fumonisin levels. The geometric mean for total aflatoxin level in the fermented product (ogi) in the two study locations (2.38 µg/kg in Ibadan and 3.20 µg/kg in Abeokuta) where the raw maize product had levels higher than LOD, were both

below the maximum tolerable limit in Nigeria (also the European Union standard) of 4 μg/kg total aflatoxin (EU, 2006). This contrasts with Okeke *et al., (*2015), who found that fermentation reduced levels of aflatoxins to below LOD. If this study results, which reflect AFB1 levels in actual environment, are compared to results observed in laboratory settings by Mokoena *et al., (*2006) and Okeke *et al., (*2015), we see lower levels of mycotoxin reduction achieved by commercial food processors compared to that in laboratory settings. Nonetheless, in all these studies, aflatoxins were reduced to an extent that would mean improved food safety, particularly for infants and young children weaned onto ogi compared to unprocessed maize-based foods. This confirms the importance of exploring the effects of strategies to reduce mycotoxins, such as ogi processing, in non-laboratory environments that are more likely to reflect reality, what consumers are actually eating, and therefore the mycotoxin levels to which they are exposed.

While lactic acid fermentation is shown to be broadly effective for mycotoxin reduction in many studies, this study also explored if processing practices may impact the effectiveness of lactic acid fermentation. Higher levels of total aflatoxin and total fumonisins were recorded in maize that had been steeped for three days, compared to two days when all cities were combined; although the results did not achieve statistical significance. In terms of storage duration, we have found statistically significant differences between AFG1, FB1 and FB2 levels in maize from Abeokuta stored for less than 7 days versus maize stored for more than 7 days. However, no statistically significant differences were observed in other cities and when samples from all cities were combined. Considering the small sample sizes and low storage periods, it is not clear that our results are meaningful.

Although our study results indicate that LAB fermentation can reduce mycotoxin levels in ogi, the mechanism of how these toxins are getting reduced during fermentation is not well

understood yet. Previous studies suggest various mechanism including noncovalent binding of the toxin to cellular material such as the cell wall skeleton fractions (peptidoglycan, polysaccharides, proteins) of the LAB (Zhang & Ohta, 1991; Haskard *et al*., 2001; Peltonen *et al*., 2001). Multiple components of the bacterial cell might be involved in the binding of AFB1 and environmental conditions may affect this interaction (Turbic *et al*., 2002; Hernandez-Mendoza *et al*., 2009;). It is also assumed that LAB can release molecules during cell rupture that may prevent mold growth resulting in lower accumulation of their mycotoxins (Zinedine *et al*., 2005). Nout, (1991) suggested AFB1 might be reduced due to LAB fermentation opening up the AFB1 lactone ring resulting in its detoxification. Reduction of fumonisins by LAB is possibly due to binding of it to the cell wall components rather than covalent binding or metabolism and peptidoglycans are the most credible binding sites for fumonisins (Niderkorn *et al*., 2009). Reduction in pH due to lactic acid production may also lead to transformation of aflatoxin and fumonisin into less toxic compounds (Galvano *et al*., 2001; Shetty & Jespersen, 2006; Jard *et al*., 2011).

There are many studies suggesting that aflatoxin and fumonisin can bind to LAB, which might make the extraction of these toxins more difficult. Therefore, our data and sample preparations may contain uncertainties. Since the exact mechanism of how these toxin concentrations are reduced is unknown, it is difficult to know if toxins have been actually lost, or are temporarily bound to other elements or compounds in the food ("masked" mycotoxins) but are still bioavailable – which cannot be detected by conventional analytical methods (Falavigna *et al*., 2012; Ahlberg *et al*., 2019; du Plessis *et al*., 2020). Experiments and analyses done with samples spiked with labeled toxins would possibly give us more insight on whether these reductions of mycotoxins by LAB fermentation are materialistic.

Another limitation of our study is that we considered the entire process of ogi processing in determining changes in mycotoxin levels (raw material and final product) in the maize. Thus, the storage in diverse facilities for the ogi processors, for different lengths of time, and different steeping times, in addition to the lactic acid fermentation step, were all considered together in determining initial and final mycotoxin levels, rather than the changes in mycotoxin levels at each step of the fermentation process. In reality, this is what southwest Nigerians encounter in their ogi consumption if purchased from one of the ogi processors. However, it would also be beneficial to examine, in the future, how mycotoxin levels change at each of the individual steps in this process. Okeke *et al., (*2015) did analyze this, finding that storage for longer periods increases mycotoxin levels (which we also found in an earlier study: Liverpool-Tasie *et al.,* 2019), and that there were mycotoxin-specific effects for impact of steeping duration on mycotoxin concentrations in white vs. yellow maize.

Another limitation of our study was that the storage times for maize before processing were relatively short (2 weeks at the longest), which is true for many, but not all, ogi processors. If maize were stored for longer periods, then mycotoxin levels might increase – particularly aflatoxins – and processing would be more important to reduce them. Maize is an important staple food crop consumed all across Africa. In many parts of the continent, it is used for ogi, a porridge-like weaning food commonly used for infant/children's food or a meal for the convalescing. Ogi is produced through lactic acid fermentation of maize.

This study attempted to explore the extent to which lactic acid fermentation of maize could reduce the level of mycotoxins (aflatoxins and fumonisins) in ogi, collected from different commercial ogi processors in southwest Nigeria. We found that, where initial levels of maize were not below the limit of detection for aflatoxins or fumonisins, both groups of mycotoxins were

reduced (significantly for fumonisins) through lactic acid fermentation processing to form ogi. In particular, the significant reduction of fumonisin through ogi processing by ogi processors represents an interesting new finding for commercial lactic acid fermentation processes in reducing an important mycotoxin in Nigerian maize. However, more exposure reduction studies are required to explore the effects of LAB on the bioavailability of aflatoxin and fumonisins in maize before this can be recommended as a public health intervention.

CHAPTER FOUR: Estimation of dietary tolerable daily intake (TDI) for non-carcinogenic effects of aflatoxin

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Abstract

Aflatoxins are toxic chemicals produced by the fungi *Aspergillus flavus* and *A. parasiticus*. In warm climates, these fungi frequently contaminate crops such as maize, peanuts, tree nuts, and sunflower seeds. In many tropical and subtropical regions of the world, populations are co-exposed to dietary aflatoxin and multiple infectious pathogens in food, water, and the environment. There is increasing evidence that aflatoxin compromises the immune system, which could increase infectious disease risk in vulnerable populations. Our aim was to conduct a dose-response assessment on a non-carcinogenic endpoint of aflatoxin: immunotoxicological effects. We sought to determine a non-carcinogenic tolerable daily intake (TDI) of aflatoxin, based on the existing data surrounding aflatoxin and biomarkers of immune suppression. To conduct the dose response assessment, mammalian studies were assessed for appropriateness of doses (relevant to potential human exposures) as well as goodness of data, and two appropriate mouse studies that examined decreases in leukocyte counts were selected to generate dose response curves. From these, we determined benchmark dose lower confidence limits (BMDL) as points of departure to estimate a range of TDIs for aflatoxin-related immune impairment: 0.017-0.082 μg/kg bw/day. As aflatoxin is a genotoxic carcinogen, and regulations concerning its presence in food have largely focused on its carcinogenic effects, international risk assessment bodies such as the Joint Expert Committee on Food Additives (JECFA) have never established a TDI for aflatoxin. Our work highlights the importance of the non-carcinogenic effects of aflatoxin that may have broader public health impacts, to inform regulatory standard-setting.

Key words: Aflatoxins, immunotoxicity, safety level, dose-response assessment, benchmark dose modeling

1. Introduction

Aflatoxins are toxic secondary metabolites of the fungal species *Aspergillus flavus* and *A. parasiticus.* They are an ongoing concern in global food safety, as they are especially common in maize and peanuts, which are staple foods in many parts of the world (Wu *et al*., 2014). Aside from maize and peanuts, aflatoxin is a common contaminant in tree nuts such as almonds and pistachios, as well as cottonseed and sunflower seeds. Aflatoxin exposure is more common in tropical/subtropical regions of the world, where high temperatures and frequently warm and wet storage conditions favor fungal growth and mycotoxin (fungal toxin) production.

Over the last 60 years, aflatoxin exposure has been associated with multiple adverse health effects. The International Agency for Research on Cancer (IARC) classifies "naturally occurring mixes of aflatoxins" in food as a Group 1 human carcinogen (IARC, 2002). The most common and toxic derivative of aflatoxin, B1 (AFB1), is metabolized in the liver into a reactive exo-8,9-epoxide form by cytochrome P450 enzymes. This exo-epoxide can bind to DNA and cause mutations that increase liver cancer risk (Kensler *et al.,* 2011). For the roughly 350 million people worldwide who are chronically infected with hepatitis B virus (HBV), the risk of aflatoxin-related liver cancer becomes synergistic (30 times higher), compared to individuals not infected with HBV (JECFA, 1998; Groopman *et al.,* 2008; JECFA, 2016). Aflatoxin exposure through maize and peanuts alone was estimated to cause 25,200 to 155,000 cases of liver cancer every year (Liu and Wu, 2010). However, although aflatoxin exposure has most frequently been associated with cancer, it is now well recognized that aflatoxin can cause many other adverse effects. At extremely high doses in maize, aflatoxin has caused acute liver failure and even death in humans from

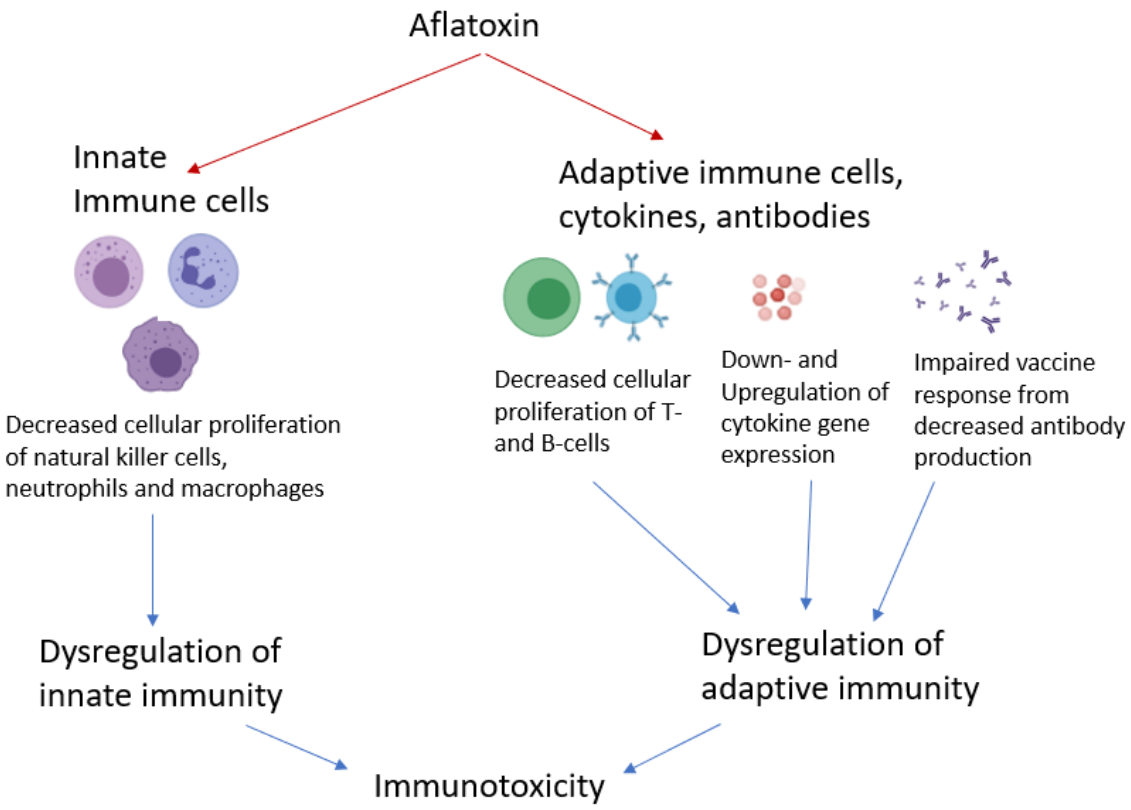
aflatoxicosis (Strosnider *et al.,* 2006). Aflatoxin is also associated with child growth impairment, pregnancy loss, premature birth, and immunotoxicity (Bondy & Pestka, 2000; Khlangwiset *et al.,* 2011; Wild et al., 2015; Smith *et al.,* 2017).

Because food safety regulations in the United States and worldwide have frequently prioritized cancer prevention, the aflatoxin regulations in over 100 nations worldwide are typically based on reducing liver cancer risk (Wu *et al.,* 2013). However, since the 1970s, a plethora of studies have linked aflatoxin to immunotoxicological effects, including several with clear dose-response relationships. Simplistically, the immune system has two main types of response: the innate response and the acquired or adaptive response. The innate immune system, which is either non-specific or broadly specific, is the primary defense against infections or antigens. Macrophages, neutrophils, monocytes, basophils, eosinophils, and natural killer (NK) cells are key cellular components of the innate immune system that work together to ensure host resistance to infections. These, for example, coordinate to repair a cut on the skin to prevent infection. The innate immune system also aids the adaptive immune system, which makes antibodies to protect the host against specific antigens in the event of future infections. Adaptive immunity is highly specific to particular pathogens in the means by which it can eliminate infections efficiently, and is triggered by vaccines or by past infections (Murphy and Weaver, 2018).

The studies relating aflatoxin to immunotoxicity have ranged from impacts to innate and adaptive immunity. These studies have been conducted across multiple species, including humans, rodents, pigs, birds, and fish. The different immunological endpoints that have been measured in these studies include immune cell proliferations, regulation of cytokine gene expression, antibody production, and host resistance to infections. In brief, these studies have indicated that aflatoxin exposure can affect macrophages, neutrophils, and NK cell-mediated functions (Reddy and

Sharma, 1989; Neldon-Ortiz and Qureshi, 1992; Cusumano *et al.,* 1996; Silvotti *et al.,* 1997; Moon *et al.,* 1999). Additionally, aflatoxin exposure was found to decrease T- and B-cell activities, which are the cellular components of adaptive immunity (Richard *et al.,* 1978; Reddy *et al.,* 1987; Hinton *et al.,* 2003). The B-cells produce antibodies and memory cells to prevent secondary infections from the same agent. T-cells play a crucial role in the adaptive immune system by helping B-cells to produce antibodies against pathogens. Some T-cells are also crucial for immune response against tumors and intracellular pathogens such as viruses. Cytokines are signaling proteins secreted by immune cells that activate target tissues and immune cells to enable more efficient responses against pathogens through amplification of immune signaling (Murphy and Weaver, 2018). Cytokines are often designated as either pro-inflammatory or anti-inflammatory. There are multiple studies that indicate aflatoxin exposure can alter expression of both pro- and anti-inflammatory cytokines (Hinton *et al.,* 2003; Meissonnier *et al.,* 2008; Qian *et al.,* 2014; Jiang *et al.,* 2015; Ishikawa *et al.,* 2017; Holsapple *et al.,* 2018; Shirani *et al.,* 2018; Wang *et al.,* 2018). Some animal studies have also demonstrated that aflatoxin may decrease host resistance to infectious diseases (Hamilton and Harris, 1971; Edds *et al.,* 1973; Wyatt *et al.,* 1975; Joens *et al.,* 1981) and result in decreased immunity to vaccinations such as Turkey herpesvirus (HVT), Newcastle disease virus (NDV) Texas GA strain, and infectious bronchitis virus (IBV) Massachusetts serotype 1B-41 (Batra *et al.,* 1991; Azzam and Gabal 1998). Fig. 7 summarizes the different immune system parameters affected by aflatoxin leading to immunotoxicity.

Figure 7: Immune system parameters affected by dietary aflatoxin exposure.



Both innate and adaptive immunity may be compromised by aflatoxin, which has implications for global health in regions where maize and peanut consumption are high and infectious diseases is common. Created with BioRender.com.

The goal of our study is to estimate a tolerable daily intake (TDI) for aflatoxin-related immunotoxicity; hence, for the first time, establishing a health endpoint for *non-carcinogenic* effects of aflatoxin. The Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization and World Health Organization has never estimated a TDI for aflatoxin in its decades of operation, although it has estimated TDIs for other mycotoxins as well as multiple

other food contaminants; perhaps because the focus of aflatoxin from a global regulatory standpoint has been cancer. TDIs are typically established for non-carcinogenic effects of chemicals and toxins. Because aflatoxin is a genotoxic carcinogen, regulations on its tolerable levels in food worldwide have been based on minimizing its presence subject to economic and technological feasibility. Here, we estimate a TDI for aflatoxin that may have regulatory relevance, and certainly has health relevance worldwide; particularly as any immunotoxin in the diets of populations highly exposed to infectious agents would be critical to control.

2. Methods

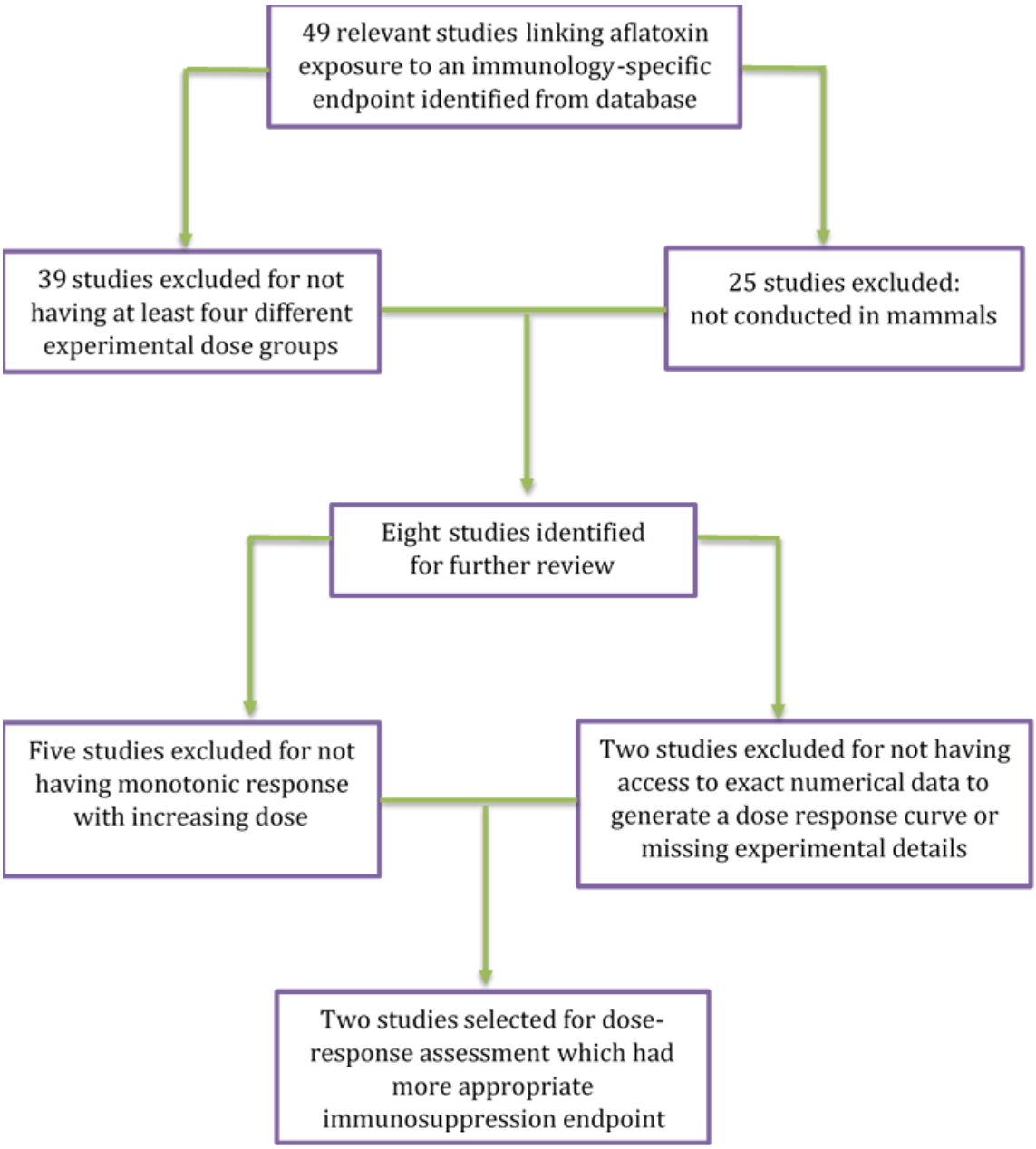
2.1 Identification of data for dose-response assessment

A review of the human studies and animal studies that have associated aflatoxins with immunotoxicity was conducted using PubMed and Google scholar, using a combination of subject headings and free text words including: aflatoxin and immune system, aflatoxin and immune suppression, aflatoxin and innate immune system, aflatoxin and adaptive immune system, aflatoxin on T-cells and B-cells, aflatoxin and vaccination. From our initial search, we found 49 dose-response studies (8 human and 41 animal studies) based on these selection criteria: 1) a mammalian study, 2) at least four different doses of aflatoxin tested, 3) monotonically increasing or decreasing adverse health effects in response to increasing doses of aflatoxin, and 4) availability of numerical data. Fig. 8 presents the process of selection of studies used for our dose-response assessment and TDI calculations. Out of the 49 studies, 39 did not have at least four different experimental dose groups, and 25 were not mammalian studies. We narrowed to seven key studies with dose-response data, of which four studies were excluded for not having monotonic responses with increasing aflatoxin doses. For the dose response assessment, there were only three available

studies that fulfilled all the above criteria and had numerical data rather than data in the form of graphs. We finally selected two studies that comparatively had more relevant endpoints of immune suppression.

Based on our review, we selected two dose-response studies to perform dose-response assessments on aflatoxin-related immunotoxicity. The first study was Reddy *et al.* (1987); in which CD-1 male mice were orally fed with 0, 0.03, 0.145 or 0.70 mg AFB_1 /kg BW every other day for two weeks and the peripheral white blood cell (WBC) counts were analyzed after two weeks of treatment. The second study was Reddy and Sharma (1989); in which seven-week-old Balb/c mice received 0, 0.03, 0.145 and 0.70 mg AFB1/ kg BW every other day for four weeks through oral gavage in corn oil and the peripheral WBC counts were measured. Both these study results demonstrated WBC levels reducing significantly ($P<0.001$) in mice treated with higher doses of aflatoxin compared to the control group indicating that higher doses of aflatoxin may result in lower WBC counts which may make infectious disease outcomes worse as they play major role in phagocytosis and defense against infection.

Figure 8: Selection of studies for inclusion in dose-response assessment TDI calculation for aflatoxin.



2.2 Dose-response analysis and TDI calculation

To estimate a tolerable daily intake (TDI) from dose-response curves, past assessments have used dose points such as the no observed effect level (NOEL; or no observed adverse effect level,

NOAEL) or the lowest observed effect level (LOEL; or lowest observed adverse effect level, LOAEL). But to make full use of the dose-response curve, rather than just one point such as the NOEL, a TDI may be calculated based on the benchmark dose (BMD) approach, which is applicable to all non-carcinogenic toxicological effects. The BMD approach uses all the dose-response data to estimate the shape of the overall dose-response curve for an endpoint. It also provides a quantification of the uncertainties in the dose-response data (EFSA, 2016). From this statistical model, the dose that corresponds to a 10% response in the test animals is identified. To account for sample variance, the 95% lower confidence limit at the BMD, which is the BMDL, is selected.

To determine these values for our selected studies, we used the Benchmark Dose Software (BMDS) version 3.2 of the United States Environmental Protection Agency (EPA). We used a continuous dose-response model with log normal distribution and 10% relative change specification as the benchmark response to generate $BMDL_{10}$ (10% change from controls). The $BMDL_{10}$ is then divided by the product of all of the applicable uncertainty factors (UF) to calculate the TDI. Here, we have used two uncertainty factors accounting for intra-species variability and inter-species variability for a composite UF = 100 (WHO, 1992).

3. Results

The dose response curves based on the study results (Table 12) were generated using the BMDS software, and the $BMDL_{10}$ values were identified from the best selected models (recommended by the software based on the lowest akaike information criterion (AIC) value) (Fig. 9). In Table 11, the aflatoxin doses (leftmost column) administered to the mice represent doses that are within the appropriate range for doses relevant to humans in different parts of the world. Decreases in white blood cell (WBC, leukocyte) counts indicate increased immune impairment.

The TDI for aflatoxin was calculated by dividing the $BMDL_{10}$ values with the composite UF of 100 for interspecies and intraspecies variability:

$$TDI = BMDL_{10}/UF$$

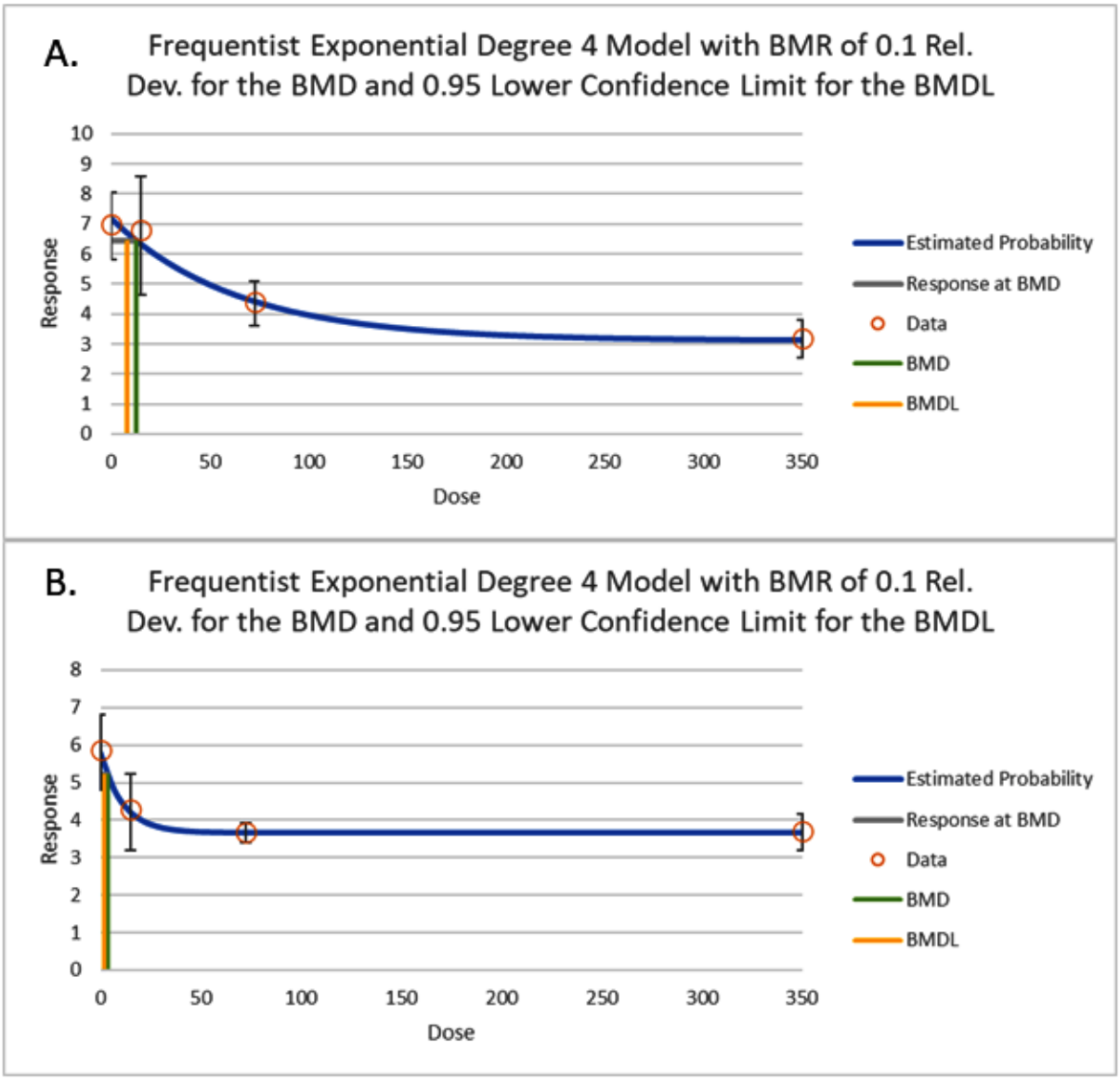
For Reddy et al. (1987) study (dose-response curve A, Fig. 3), the yellow line represents the $BMDL_{10}$ = 8.18 µg/kg bw/day. Hence, $TDI_{(Reddy\ et\ al.,\ 1987)}$ = 8.18/100 = 0.082 µg/kg bw/day. For Reddy and Sharma, 1989 study (dose-response curve B, Fig. 3), the yellow line represents the $BMDL_{10}$ = 1.74 µg/kg bw/day. Hence, $TDI_{(Reddy\ and\ Sharma,\ 1989)}$ = 1.74/100 = 0.017 µg/kg bw/day. Based on the dose-response curves generated from these study results, we estimate a range of TDI for aflatoxin related immunosuppression to be 0.017-0.082 µg/kg bw/day.

Table 12: **Effects of different doses of aflatoxin on white blood cell (WBC) counts in mice from two studies: Reddy et al. (1987), and Reddy and Sharma (1989).**

Aflatoxin dose (µg/kg bw/day)	**WBC/mm^3 (x10^{-3}) Reddy et al., 1987**	**WBC/mm^3 (x10^{-3}) Reddy and Sharma, 1989**
0	7	5.86
15	6.8	4.3
72.5	4.4	3.67
350	3.2	3.7

Note: The mice were exposed to AFB1 every other day. Therefore, to get "per day exposure dose" we divided the doses by two.

Figure 9: Dose-response curves from BMDS software.



Note: A – dose response curve generated using Reddy et al. (1987) study results; BMDL= 8.18 µg/kg BW/day. B – dose response curve generated using Reddy and Sharma (1989) study results; BMDL = 1.74 µg/kg BW/day. The x-axes represent the dose (µg aflatoxin / kg BW/day) and y-axes represent the mice peripheral WBC count (WBC/mm3 ($x10^{-3}$)). The yellow line represents the $BMDL_{10}$ values.

4. Discussion

Aflatoxin exposure is especially concerning for people living in developing countries in warm regions of the world, such as certain Sub-Saharan African countries where maize and peanuts are consumed as staple foods (Liverpool-Tasie et al. 2019). The combination of high consumption of these foods, and conducive climates for fungal growth and toxin production in those foods, leads to higher exposure to dietary aflatoxin. Because at-risk populations worldwide are frequently co-exposed to aflatoxin and infectious pathogens, it is critical to understand the possible effects of aflatoxin on the immune system.

As aflatoxin's carcinogenic effects have been known for nearly 60 years and have been the focal point for food safety regulations worldwide, international risk assessment bodies such as JECFA have not established non-carcinogenic TDIs for aflatoxin. However, since there is substantial evidence in the literature that aflatoxin has potential immunosuppressive effects, it is important that the non-carcinogenic risks of aflatoxin are also considered by policy makers when setting regulations for controlling this food contaminant. Moreover, lack of setting any TDI for aflatoxin has left governing bodies at somewhat of a loss to set maximum tolerable levels for this toxin. Since the oft-challenged maxim is that there is no safe level of a genotoxic carcinogen, regulations have often been based on levels "as low as reasonably achievable," with some attention to the economic and technological feasibility of setting low maximum tolerable levels. Providing evidence for aflatoxin's non-carcinogenic effects and estimating a TDI based on these will provide policy makers guidelines for the purposes of preventing adverse health effects besides cancer, which – in the case of aflatoxin-related immune system dysfunction – may be more immediately critical in many parts of the world.

This is, to our knowledge, the first estimation of a TDI of aflatoxin's immunotoxicological effects. Based on an intensive literature review, we have selected two dose-response studies; and based on those studies, we have determined 0.017-0.082 µg/kg bw/day to be the range of TDI for aflatoxin related immunosuppression. When extrapolating from a TDI to a maximum tolerable limit in food, it is important to note that we estimated these TDIs for AFB1 alone. AFB1 levels are approximately half of total AF levels (B1 + B2 + G1 + G2). Based on our calculations, it could be estimated that dietary exposure to over 0.017 µg/kg bw/day AFB1 may reduce the peripheral WBC counts in humans. This estimation is relatively high compared to the average daily intake of aflatoxin in Europe (0.00093–0.0024 µg/kg bw/day) and United States (0.0027 µg/kg bw/day) but falls in the range of that in Asia (0.0003–0.053 µg/kg bw/day) and in Africa (0.0035–0.18 µg/kg bw/day) (JECFA 2007). This has harmful immunological implications; since WBC play major roles in the innate immune response which include rapid protection from microbial pathogens, removal of foreign antigens, and presentation of antigens to the adaptive immune system for further protection and the prevention of secondary infections (Gordon-Smith, 2013).

We acknowledge that our TDI estimation has limitations since we have used data from old studies. Today, more sensitive methods and techniques exist which might provide a different and more accurate BMDL values from similar dose-response studies. Another limitation would be, the two mice strains used in the Reddy *et al.* 1987 and Reddy and Sharma, 1989 studies were CD-1 and BALB/c mice which are more likely to tolerate aflatoxin better compared to Fischer rat strains (Choy, 1993). Lower BMDL values might be obtained if the study was done in Fishcher rats. Therefore, future studies are required to reproduce and confirm the dose-response data observed in the studies by Reddy et al. (1987, 1989). Also, similar dose response studies are necessary to explore other immunological endpoints, such as, cytokine production, lymphocyte counts,

antibody production in response to vaccines etc., in response to aflatoxin exposure. This would help to confirm whether the TDI calculated in this report is safe enough to prevent aflatoxin-induced immunosuppression or if a stricter TDI is warranted for immunological protection.

CHAPTER FIVE: Quantitative risk assessment of immunotoxic risk of aflatoxin in Southwest-Nigerian children and adults

Abstract

Nigeria has an extremely high rate of infectious disease and associated mortalities in children under five years old. Therefore, it is very important to control any environmental agent that could impair child immunity. Aflatoxin, a mycotoxin produced by *Aspergillus* species in variety of food, is well-known to be hepatocarcinogenic and its carcinogenic risk has long been investigated in many parts of the world. However, limited epidemiological studies and numerous animal studies have also indicated that aflatoxin is immunotoxic but the non-carcinogenic risk of aflatoxin has never been assessed. Our preliminary work in Oyo State, Nigeria, shows that maize stored in homes for human consumption frequently contains dangerously high levels of aflatoxin. In this study we examined the immunosuppressive risk from dietary aflatoxin exposure in Southwest-Nigerian infants and adults based on their daily dietary exposure to aflatoxin through maize and groundnut consumptions. The results of our quantitative risk assessment suggest that infants and children of age 6 months to 3 years old living in the rural sector of southwest Nigeria are at reasonable to great risk for aflatoxin-induced immunosuppression (hazard quotient (HQ) values: 12.42 – 74.2); there is a possible to reasonable chance of risk for rural adults (HQ = 3.55 to 10.6). The HQ values (1.97 – 3.93) for infants and children living in the urban sector suggest a possible risk of immunosuppression from dietary aflatoxin exposure. However, the dietary aflatoxin exposures in adults living in the urban sector are not high enough to cause immunosuppression (HQ < 1). This calls for adequately addressing and regualting aflatoxins more strictly in Nigerian maize and groundnuts, especially in the rural sectors to protect child immunity.

Key Words: Aflatoxin, risk assessment, immunosuppression, Southwest-Nigeria, children

1. Introduction

Aflatoxins are secondary metabolites of the fungi *Aspergillus flavus* and *A. parasiticus*, which widely contaminate many staple foods and cause a broad range of adverse health effects in both animals and humans (Eze*et al*., 2018; Alshannaq and Yu, 2017; Wu *et al*., 2014; Shephard, 2008). Aflatoxin contamination of food is a serious global food safety concern. These mycotoxins often contaminate maize and groundnuts, mainly in tropical and sub-tropical countries where the fungal growth and mycotoxin production are favored by the high temperatures and warm and wet storage conditions. In high-income countries aflatoxins are regulated strictly, however the aflatoxin regulations in crops are not implemented as strictly in developing nations which results in chronic exposure to aflatoxins in humans (Shephard, 2003, Williams *et al*., 2004). The US Food and Drug Administration (US FDA) has set a limit of 20 μg/kg of aflatoxins in foods that are meant for human consumption (US FDA 2000). European Union (EU) has a much stricter limit for aflatoxins: 2 μg/kg AFB1 and 4 μg/kg total aflatoxins for nuts and cereals for human consumption (European Commission 2010).

There are four major types of aflatoxins: AFB1, AFB2, AFG1, and AFG2. AFB1 is the most common contaminant in food, and the most toxic. The International Agency for Research on Cancer (IARC) has classified "naturally occurring mixture of aflatoxins" as a Group 1 human carcinogen (IARC 2002). Numerous epidemiological and animal studies show that aflatoxin contributes to causing hepatocellular carcinoma (HCC); for individuals who are simultaneously infected with chronic hepatitis B virus (HBV) infection, the risk of aflatoxin-related liver cancer is roughly thirty-fold higher due to a possible synergistic interaction between HBV infection and the mutagenic capacity of aflatoxin (JECFA 1998; Moudgil *et al.*,2013; Wu, Stacey & Kensler 2013). Dietary exposure to aflatoxins at high doses is associated with acute aflatoxicosis, acute

liver damage, edema, and even death (Azziz-Baumgartner *et al.*,2005; Strosnider *et al.*,2006). Other adverse health effects associated with aflatoxin exposure includes growth impairment and stunting in children (Khlangwiset *et al.*,2011; McMillan *et al.*,2018), reproductive toxicity (Agnes and Akbarsha 2003; Supriya and Reddy 2015), pregnancy loss and premature birth (Smith *et al.*,2017) and immunotoxicity (Bondy and Pestka 2000; Mohsenzadeh *et al.*,2016; Appendix A).

In many parts of the developing world, especially sub-Saharan Africa, populations face co-exposure to dietary aflatoxin and multiple infectious agents in food, water, and the environment. Hence, to the extent that aflatoxin may impair human immune responses, it is critical to understand immunity in these populations, particularly among children. Nigerian diet contains maize and groundnuts which are the two main commodities most prone to aflatoxin contamination. The maize is consumed in many different ways: on the cob (boiled or roasted), wet or dry cereal, steamed custard, pudding, porridge, and maize gruel (Ademola *et al.*, 2021). The groundnuts are consumed as boiled and roasted as a quick snack and it is also used in processed form for sauces, as a paste eaten as a side dish and as a condiment for roasted meat (suya). The Standards Organization of Nigeria (SON) has set standards for maximum total aflatoxin concentrations in maize and groundnuts for 4 μg/kg (SON, 2008). However, despite of having set regulations, our previous study analyzing aflatoxin concentrations in Southwest Nigerian maize found aflatoxin to be a prevalent contaminant of maize for human consumption (Liverpool-Tasie and Saha Turna *et al.*,2019). In 2019, Nigeria had the highest numbers of deaths for children under 5 years old (WHO 2020); most of these deaths are caused by infectious disease. Thus, it is important to control environmental agents that could impair child immunity.

The purpose of this study was to investigate whether there is a risk from a non-carcinogenic endpoint of aflatoxin, through immunological effects, in Southwest Nigeria. In our previous study

(Chapter 4), we have determined a range for tolerable daily intake (TDI) for AFB1 (0.017 – 0.082 μg/kg BW/day) based on two mice dose-response studies by Reddy *et al., (*1987) and Reddy and Sharma (1989). Both the mice studies indicated that exposure to AFB1 may lower WBC counts which play major role in phagocytosis and defense against infection. We will be using the most precautionary TDI from our previous study (Chapter 4) to conduct a quantitative risk assessment in Southwest Nigerian children (Age: six months to three years) and adults living in both urban and rural sectors, based on their daily maize and groundnut consumptions. This is the first quantitative risk assessment study that has ever explored the aflatoxin-induced immunosuppression in Nigeria or in any other country.

2. Materials and Methods

2.1 Data Collection for Aflatoxin Concentrations in Maize and Groundnuts

We have conducted the exposure assessment separately for the rural and urban sectors in Southwest Nigeria because typically Urban dwellers do not have the same dietary patters as their rural counterparts due to the differences in dietary preferences and dependence on purchased food because rural dwellers mostly consume the food, they produce themselves. Also, because of Bennett's law, one would expect that households in urban areas might consume more diversified diets (and thus less starchy staples such as maize) because of higher incomes.

Here, we have considered only the AFB1 levels rather than total aflatoxins (AFB1+AFB2+AFG1+AFG2) because the TDI values were estimated for AFB1 alone (Chapter 4). Also, AFB1 is the main aflatoxin that is expected to have the immunological effects based on previous immunotoxicity studies in the literature (Appendix 1) and the levels of the other aflatoxins are comparatively much lower than AFB1 in contaminated food. For AFB1

concentrations in maize, we have used data from our previous study (Chapter 2), where the occurrence of AFB1 in addition to AFB2, AFG1 and AFG2 levels were analyzed in maize samples collected from farmers' fields and stores and also market samples from maize traders. For the exposure assessment, the average AFB1 concentration determined in samples collected from farmers was used for the rural population and the average AFB1 concentration determined in market maize samples from maize traders was used for the urban population.

Since groundnuts, which are frequently contaminated with aflatoxins, are also significantly consumed in Nigerian, we have taken aflatoxin contamination in groundnuts into account for the exposure assessment. However, we have not personally collected groundnut samples to determine the aflatoxin concentrations in them. PubMed and Google Scholar search engine databases were searched using the key words: [aflatoxin], [AFB1], [groundnuts], [occurrence], [Nigeria] to find studies that reported AFB1 levels in Nigerian groundnuts published after 2010. Four studies were identified that reported AFB1 levels in groundnuts collected from markets located in Southwest Nigeria. For each study, the samples that had non-detectable levels of AFB1 was taken into account by assuming the minimum value to be half of the LODs reported, and the mean was calculated using the following equation:

Mean for AFB1-positive and AFB1-negative samples combined = [(Mean)*(percentage of positive samples)] + [(LOD/2) *(percentage of negative samples)]

The geometric mean value of the average AFB1 levels reported or calculated from these studies was used to estimate the average AFB1 concentration in groundnuts purchased by the urban population in Southwest Nigeria. We were unable to find aflatoxin concentration data in groundnuts from fields and household storage in Southwest Nigeria. However, majority of

groundnuts that is consumed in the south of Nigeria is not produced there but comes from the northern parts of the country. Therefore, to estimate the AFB1 exposure from groundnut consumption in rural setting, we considered a range of ratios including assuming the same levels in rural and urban areas and also a similar ratio for storage AFB1 accumulation in rural vs urban maize that we observed in our previous study (Chapter 2).

2.2 Food Consumption Data

The average daily dietary consumption data in adults for maize and groundnuts in both urban and rural sectors of Southern Nigeria were obtained from the most recent available version of the Nigeria Living Standards Measurement Study–Integrated Survey on Agriculture (LSMS-ISA) by the World Bank for Nigeria from 2018/2019. For infants and children (Age 6 months to 3 years), the consumptions values by adults were divided by half, assuming children in that age range would consume approximately half of the amount of maize and groundnuts that an average adult would consume (assumption based on personal communication with Ms. Ademola and Dr. Wu). The LSMS-ISA dataset is nationally representative and also at different geopolitical zone level which includes maize, maize flour and groundnut consumption information collected at the rural and urban household level in a period of one week across both Southern and Northern Nigeria. The LSMS-ISA implements the General Household Survey (GHS), which is carried out throughout the country in February-March on 5,000 households which are a subsample of the GHS core survey of 22, 000 households to produce state level estimates.

2.3 Exposure Assessment

The average daily doses (ADD) of AFB1 from maize and groundnut consumptions by Southwestern Nigerian children and adult population residing in both rural and urban sectors were

calculated based on the concentrations on Table 13 and the average intake rate by using the formula:

$$ADD = (C_{ave} * IR_{ave})/BW$$

where ADD = average daily dose, IR_{ave} = daily average Intake Rate (kg/day), C_{ave} = average concentration of aflatoxin in maize and groundnuts (μg/kg), and BW = body weight of the global-average adult: 70 kg and of an average child (age: 6 months – 3 years): 10 kg.

2.4 Risk Characterization

The final step of the risk assessment determines whether an individual may suffer from an adverse health effect from dietary aflatoxin exposure based on whether their average daily dose (ADD) is higher than the tolerable daily intake (TDI), above which it may potentially cause adverse effects. If ADD > TDI, then a potential health risk exists (WHO, 2009). This is done by calculating the Hazard quotient or HQ where the ADD of AFB1 is divided by the TDI of AFB1 (determined in Chapter 4). If the HQ value is much more than one (>>1), that would imply a great risk, if HQ value is slightly more than one (>1), that would imply a possible risk, and if HQ is less than one (<1), that would imply there is no immunosuppressive risk from dietary aflatoxin exposure.

For our risk assessment, we have assumed the daily average intake rate of maize and groundnuts by infants and children to be half of the amounts that an adult would consume. However, previous Nigerian risk assessment studies in children, on the carcinogenic effects of aflatoxin, have used the same dietary consumption values (IR_{ave}) of maize and groundnuts by adults (Oyedele *et al.*,2017; Adetunji *et al.*,2018). To be consistent to the available literature, we have also calculated HQ values with assumptions that infants and children (6 months to 3 years old) consume the same amounts of maize and groundnuts as adults.

3. Results

Table 13 summaries the average AFB1 levels in Southwest Nigerian maize and groundnuts found in both urban and rural sectors. We found some samples containing very high levels of aflatoxins in farmers' maize that were stored for longer than two months (Chapter 2). This resulted in the average AFB1 content in farmers' maize, representing the exposure in rural population, to be very high: 39.80 µg/kg – exceeding both the EU and Nigeria standards, as well as the US FDA limits set for aflatoxins in maize. However, the average AFB1 level in maize samples collected from the markets was 3.10 µg/kg, which was within the aflatoxin standards set by the regulatory bodies.

In all the four studies identified that reported aflatoxin concentrations in groundnut samples collected from different markets in Southwest Nigeria, indicate the mean AFB1 levels to be higher than the EU and Nigerian standards of 4 µg/kg. The average AFB1 level in groundnuts combining the average values in all four studies, was found to be 24.03 µg/kg. Since all these samples were collected from different markets located in Southwest Nigeria, the combined average AFB1 concentration is representing the exposure in urban populations. Compared to the lower aflatoxin levels in maize collected from markets in urban areas, the aflatoxin contaminations in groundnuts from markets were very high. To represent the AFB1 exposure from groundnut consumption in rural sectors, the mean AFB1 level in market groundnut samples was multiplied by 12.4, assuming a similar ratio for AFB1 accumulation in rural vs urban maize, which came up to 309 µg/kg which is extremely high and almost close to causing acute toxicity in humans; aflatoxin exposure >400 ppb is associated with causing acute liver failure in humans (Azziz-Baumgartner *et al.*,2005, Strosnider *et al.*,2006).

Table 13: AFB1 levels in Southwest Nigerian maize and groundnuts.

Study	Ayejuyo *et al.*,2011	Afolabi *et al.*,2014	Oyedele *et al.*,2017	Adetunji *et al.*,2018	Liverpool-Tasie and Saha Turna *et al.*,2019 (Chapter 2)	
Type of sample	Groundnuts	Raw and roasted groundnuts	Raw shelled groundnuts	Raw groundnuts	Maize cobs	Maize grain
Location	Lagos	Lagos, Ogun, Oyo	Derived Savanna	Ogun	Oyo State, Atisbo and Saki West	Greater Ibadan area of Oyo State
Sample Source	Major markets	Markets	Major markets where groundnuts are sold in bulk quantities	Major markets	Farmers' fields and stores	Major maize wholesale markets
Method	ELISA	HPTLC	LC MS/MS	ELISA	LC MS/MS	
Number of samples	24	48	32	15	71	15
Mean of AFB1 conc. (µg/kg)	5.85	50.31	17.21	32.26	39.80	3.10

Combined geomean AFB1 conc. (C_{ave}) *(µg/kg)*	Rural groundnuts*	Urban groundnuts	Rural maize	Urban maize
	20.10 or 258.1	20.10	39.80	3.10

*Note: For rural groundnuts, C_{ave} is either same as urban groundnuts (20.10 µg/kg) or 258.1 µg/kg, which is determined using the same ratio of AFB1 accumulation in rural vs urban maize (Liverpool-Tasie and Saha Turna *et al.*,2019): 39.8/3.1 = 12.4; 24.03 µg/kg was multiplied with 12.4 to get an estimation of AFB1 level in groundnuts in rural sector.

Table 14 shows the IR_{ave} values and ADD values of AFB1 from maize and groundnuts which are calculated using the ADD equation described above. The highest average daily exposure to AFB1 (0.630 µg/kg BW/day) was observed in infants and children residing in the rural sector. This might be due to the higher consumption amounts of both groundnuts and maize among the rural infants and children and also the AFB1 contaminations being significantly higher in the rural maize and groundnuts compared to that in the urban sector. The average daily exposures to AFB1 in

adults residing in rural sector was higher (0.180 µg/kg BW/day) compared to that in adults residing in the urban sector (0.010 µg/kg BW/day).

Table 14: Dietary exposure to AFB1 in Southwest Nigeria

	IR_{ave} (kg/day)			
	Rural* children	Urban* children	Rural adults	Urban adults
Maize	0.044	0.028	0.088	0.056
Groundnuts	0.018	0.012	0.035	0.025
ADD (µg/kg BW/day)	**0.211-0.630**	**0.033**	**0.06-0.180**	**0.010**

Note: * = Half of IR_{ave} for adults

Average Intake Rate (IR_{ave}) values are obtained from the Nigeria Living Standards Measurement Study–Integrated Survey on Agriculture (LSMS-ISA) 2018/2019 where the data was reported as amount consumed in a week per household. To get "per day/per capita" intake rate, each of the value was divided by 7 and then divided by the number of people living in that household. The IR_{ave} here is the average of the consumption amounts for all households.

Average Daily Dose (ADD) values are calculated using the equation: $ADD = (C_{ave} * IR_{ave})/BW$, representing AFB1 exposure from both maize and groundnuts

The average body weight (BW) estimations were 10 kg for 6 months to 3 years old and 70 kg for adults

ADD values for rural population are in ranges since we have two different values for C_{ave} (average AFB1 concentration) for rural groundnuts

The Hazard Quotients (HQ) (Table 15) for infants and children living in the rural sector with both half and same adult IR_{ave} were found to be much higher than 1 (HQ_{half} = 12.42 to 37.1 and HQ_{same} = 24.84 to 74.2). This implies that there is great risk of immunosuppression from the AFB1 exposure that the infants and small children in the rural sector are getting from their daily maize and groundnut consumptions. For infants and children living in the urban sector, the range of HQ values is 1.97-3.93, indicating a chance of possible risk for aflatoxin-induced immunosuppression. For adults, tthe HQ range for rural sector (3.55 to 10.6) indicated a possible to reasonable chance of risk and for urban sector (0.56), it implied no risk of aflatoxin-induced immunosuppression

based on the amount of dietary AFB1 exposure they get from their daily maize and groundnut consumptions. For both rural children and rural adults, the HQ values are much higher than that in the urban sector. This was expected as both the AFB1 contamination and maize and groundnut consumption were much higher in the rural areas compared to the urban areas of southwest Nigeria.

Table 15: Risk Characterization of AFB1-induced immunosuppression in Southwest Nigeria

TDI = 0.017 µg/kg BW/day	**HQ Rural**	**HQ Urban**
Infants and Children (half IR_{ave} for adults)	12.42 to 37.1	1.97
Infants and Children (same IR_{ave} for adults)	24.84 to 74.2	3.93
Adults	3.55 to 10.6	0.56

TDI: Tolerable Daily Intake calculated in Chapter 4 using Reddy and Sharma, 1989 study
IR_{ave} = Daily intake rate of maize and groundnuts
HQ: Hazard Quotient

HQ_{Rural} in ranges since we had assumed two different values for C_{ave} of AFB1 in rural groundnuts (Table 13)

HQ values are calculated using the equation: HQ = ADD/TDI (ADD represents AFB1 exposure from both maize and groundnuts)

HQ >> 1.0 implies great health risk
HQ > 1.0 implies possible health risk
HQ ≤ 1.0 implies no health risk

4. Discussion

In many developing countries, aflatoxin is a common contaminant in the staple diets, and children are more sensitive to aflatoxin with higher concentrations of exposure on their body weight basis. Our preliminary work in Southwest Nigeria (Chapter 2), shows that Nigerian maize frequently has high levels of aflatoxins. The carcinogenic risk has long been investigated in many parts of the world. However, epidemiological studies and numerous animal studies have also indicated that

aflatoxin is immunotoxic but the non-carcinogenic risk of aflatoxin has never been assessed. Nigeria has an extremely high rate of infectious disease and associated mortalities in children under age 5 (Wakabi 2008). Therefore, we wanted to investigate whether there is an immunotoxic risk associated with dietary consumption of aflatoxin contaminated maize and groundnuts in Southwest Nigerian infants and children and adults.

The average AFB1 levels in both maize and groundnuts collected from rural sectors were much higher than the Nigerian aflatoxin regulation of 4 µg/kg and also the US FDA limit of 20 µg/kg (Ayejuyo *et al.*,2011; Afolabi *et al.*,2014; Oyedele *et al.*,2017; Adetunji *et al.*,2018; Liverpool-Tasie *et al.,* 2019)**.** The average AFB1 levels in the maize samples collected from markets, representing exposure to the urban population, was within the Nigerian regulatory limits (Chapter 2). Oyedele *et al.,* (2017) even found levels up to 710 µg/kg AFB1 and a maximum of 2076 µg/kg total aflatoxins in one of their groundnut samples collected from a major market that sells groundnuts in bulk. This indicates that despite of having set aflatoxin regulation standards in Nigeria, they are not enacted appropriately even in the foods that are purchased from the markets.

According to the World bank LSMS-ISA data, the consumption amounts (IR for maize and groundnuts are higher in the rural sectors compared to the urban sectors, which is reasonable because people living in urban settings have better socioeconomic status and can afford to have diversity in their diets and include other staples such as rice, sorghum, cassava in addition to maize and groundnuts. Hence, the overall dietary exposures to AFB1 are much lower in urban populations compared to the rural populations. The low HQ value for adults in the urban sector (HQ = 0.56) suggests that there is no immunosuppressive risk from the average AFB1 exposure in this population. This conclusion is for an individual consuming the average amount of maize in urban sector of South-west Nigeria, however, for households where maize is more of an important

staple (e.g., poorer urban households), the risk might be much higher. The HQ values for infants and children living in the urban sector indicate a possible risk for aflatoxin induced immunosuppression (HQ = 1.97 – 3.93). Comparatively, the HQs for infants and children residing in rural sector were much higher, 37.1 (for half adult IR_{ave}) or 74.2 (for same adult IR_{ave}), indicating a great risk of aflatoxin-induced immunosuppression in this population. The HQ value of 10.6 for adults residing in the rural sector of Southwest Nigeria also indicate a chance of risk for immunosuppression from the average dietary AFB1 exposure.

Even though we have focused our risk assessment study in the Southwest Nigeria, aflatoxin-induced immunosuppression might be of a greater concern in the people living in the Northern Nigeria due to the economic and social imbalance between the North and South of Nigeria and because of the importance of maize and peanuts in their diet which is much more than in the south. Poverty is predominant in northern Nigeria compared to the South, with two-thirds (66%) of the Nigerian poor residing in the North (World Bank 2014; Babalola and Oyenubi 2018). People living in northern Nigeria may have much lower dietary diversity and according to the LSMS-ISA data by the World Bank, the average intake rates of both maize and groundnuts by Northern Nigerian residents are significantly greater compared to Southern Nigerian residents. Also, peanuts are largely produced in the north and their staple is more of cereals (such as maize) compared to the south. This suggests that the dietary aflatoxin exposure in humans is likely to be much higher in the northern Nigeria.

In fact, Oyedele *et al., (*2017) has reported the AFB1 levels in groundnuts collected from markets in Northern agroecological zones to be 97 µg/kg (calculated mean), which is much higher than the average AFB1 level in the Southwest (17.2 µg/kg). Another study reported the mean AFB1 levels in groundnuts collected in Niger state to be 53.06 µg/kg (Ifeji *et al.*,2014). In terms

of maize, Adetunji *et al., (*2014) analyzed farmers' maize samples collected from Kano, Sokoto, Kaduna and Niger States in which the average AFB1 level was 235.7 µg/kg, which is significantly higher than what we have observed in farmers' maize samples collected from Southwest Nigeria (39.80 µg/kg) (Chapter 2). However, this study has also found very high levels of AFB1 in samples collected from the Southern agroecological zones as well (calculated average: 371.3 µg/kg) (Adetunji *et al.*,2014). The HQ value based on these studies in the North, come up to >100 for infants and children (half IR_{ave}) indicating a severe risk of immunosuppression from dietary AFB1 exposure, much greater compared to that in the children from southwest Nigeria.

Since early life exposure may impact health and diseases later in life, Nigerian children, especially who are living in Northern Nigeria and the rural sectors in Southwest Nigeria, are potentially vulnerable to the immunotoxic health effects from aflatoxin. Therefore, it is crucial to adequately address and regualte aflatoxins more strictly in Nigerian maize and groundnuts to protect child immunity.

CHAPTER SIX: Conclusions and future directions

Many developing countries, especially sub-Saharan Africa countries are susceptible to the exposure of different mycotoxins produced by crop fungi due to lack of proper surveillance. Due to their high stability, mycotoxins not only affect crop production, but also transport, storage, processing, and post-processing stages that significantly contributes to food, feed and economic losses. Moreover, the adverse health effects of mycotoxins have negative impacts on human health and livestock. Aflatoxins, produced by the filamentous fungi *A. parasiticus* and *A. flavus*, are well-known to be hepatotoxic and carcinogenic; there are also substantial evidence in the existing literature for immunotoxic properties of this mycotoxin.

In the developing nations, especially sub-Saharan African countries, populations are co-exposed to dietary aflatoxins and multiple infectious agents in food, water, and the environment. Therefore, it is important to control the exposure to any environmental agent such as aflatoxins that could potentially suppress the immune system, particularly in children. Nigeria has one of the highest under-5 mortality rates in the world and most of these deaths are caused by infectious disease. Studies also show that aflatoxin and fumonisin (another mycotoxin, produced by *Fusarium* fungi) may have additive and synergistic toxicological effects. In this dissertation, we have assessed the prevalence and co-occurrence of these two mycotoxins along the Nigerian maize value chain. We have also studied if lactic acid bacteria fermentation reduces levels of these mycotoxins in a popular cereal and weaning food in Nigeria called ogi. Finally, we have conducted a quantitative risk assessment on an immunosuppressive endpoint of aflatoxin in Southwest Nigeria. The work presented in each chapter is summarized below.

In chapter 2, we determined the extent of occurrence and cooccurrence of aflatoxins and fumonisins in the value chain of Nigerian maize and maize-based products for human

consumption. We found for both farmers' and traders' samples, the mean total aflatoxin levels to increase with the duration of storage. Some of the samples collected form farmers' storage contained alarming levels of total aflatoxins (up to >1400 μg/kg) which could potentially cause acute toxicity in humans. In terms of fumonisin levels, no particular correlation was observed with storage duration. Both total aflatoxin and total fumonisin levels were higher in the non-branded maize snacks compared to branded snacks. Eighty percent of the non-branded snacks exceeded the Nigerian regulatory limit of 4 ppb for aflatoxins. However, total fumonisin levels were below the USFDA regulatory limit of 2000 ppb in both branded and non-branded snacks. Forty-two % of the total maize samples collected contained higher than 4 ppb of total aflatoxins that would be considered harmful by either Nigerian or the US standards. The co-occurrence was at multiple stages along the maize value chain: from harvest to postharvest storage to processed food products in the marketplace. Thus, addressing the mycotoxin risk effectively requires consideration of the entire maize value chain in Southwest Nigeria.

In chapter 3, we examined the impact of lactic acid bacteria (LAB) fermentation in reducing aflatoxin and fumonisin in ogi, a popular cereal and weaning food in Nigeria. We evaluated the prevalence of aflatoxins and fumonisins in maize grain and ogi (before and after processing) obtained from commercial ogi processors located at three different states in southwest Nigeria and determined if lactic acid fermentation can significantly reduce mycotoxin levels in ogi. After processing, the mean total aflatoxin level in ogi was close to 4 μg/kg which is the maximum acceptable limit by Nigerian standards. The fumonisin levels in maize were significantly reduced by LAB fermentation performed by commercial ogi processors which is a novel finding for reducing an important mycotoxin in Nigerian maize. Since ogi is a popular weaning food for Nigerian children, the LAB fermentation process used to produce it is potentially advantageous in

reducing health risks associated with mycotoxin exposure in sensitive population. However, more exposure reduction studies are required to understand the effects of LAB on the bioavailability of these mycotoxins in maize before it can be suggested as a public health intervention.

In chapter 4, a dose-response assessment was conducted based on the existing data on aflatoxin and immunological effects and a range of non-carcinogenic tolerable daily intake (TDI) of aflatoxin was determined. Following an intensive literature review, two dose-response mice studies were selected to generate dose response curves, that examined the peripheral white blood cell counts after treating the mice with different doses of aflatoxin. Based on these study results dose-response curves were generated using the BMDS software version 3.2 (EPA website). We determined benchmark dose lower confidence limits (BMDL) as points of departure to estimate a range of TDIs for aflatoxin-related immune impairment taking two uncertainty factors into account for inter- and intra-species variability: 0.017-0.082 µg/kg bw/day. Since aflatoxin is a genotoxic carcinogen, regulations concerning its presence in food have largely focused on its carcinogenic effects. The international risk assessment agencies such as the Joint Expert Committee on Food Additives (JECFA) have never established a non-carcinogenic tolerable daily intake (TDI) for aflatoxin. This chapter highlights the importance of the non-carcinogenic effects of aflatoxin that could be very useful for public health authorities.

In chapter 5, a quantitative risk assessment of aflatoxin-related immunosuppression was conducted in Southwest-Nigerian children and adult populations based on their daily dietary exposure to aflatoxin through maize and groundnut consumptions. The hazard quotient values were calculated using the TDI value calculated in chapter 4. The results of our quantitative risk assessment suggest a great immunosuppressive risk from dietary aflatoxin exposure (HQ = 37.1 to 74.2) among infants and children (age 6 months to 3 years) who reside in the rural settings of

South-west Nigeria. The risk is comparatively lower in children living in the urban sector; however, the HQ values were greater than one which still suggests a concern for possible risk. For adults, the HQ value of 10.6 for rural population indicates a reasonable chance of risk, but the HQ value for urban population was less than one, suggesting that the dietary aflatoxin exposures in the urban adult populations are not high enough to cause immunosuppression.

Based on the findings in this dissertation, future research may consider the following measures in order to reduce mycotoxin exposures and protect human health from the associated toxicities:

- Studies are required to understand the exact mechanism of how mycotoxin concentrations are reduced during LAB fermentation. It is not clear if the toxins are actually lost, or are temporarily bound to other elements or compounds in the food ("masked" mycotoxins) and are still bioavailable – which cannot be detected by conventional analytical methods.

- The dose-response studies by Reddy *et al.*,1987 and Reddy and Sharma 1989 need to be repeated to confirm if similar results are observed by the more sensitive methods and techniques that are available today to derive more accurate BMDL values from similar dose-response studies. A more expanded dose-response curve with more doses in the low dose region need to be considered.

- More dose response studies are needed to explore other immunological endpoints, such as cytokines production, lymphocyte counts, antibody production in response to vaccines etc. This would help to confirm whether the TDI that we have determined is safe enough to prevent aflatoxin-induced immunosuppression or if a stricter TDI is necessary.

- More comprehensive data on dietary maize and peanut consumption amounts among infants and children in both Southern and Northern Nigeria should be collected to determine the exact amount of dietary aflatoxin exposure in these populations.
- Quantitative risk assessment studies based on immunosuppressive effects of aflatoxin are needed in other countries that have high dietary aflatoxin exposure (Gambia, Uganda, Kenya, Tanzania etc.) and high under 5 mortality rates.
- In many developing nations, including Nigeria, women are more likely to be exposed to higher aflatoxin levels than men, because the men typically consume more animal source foods and women consume more grains and pulses, which have more aflatoxin contamination. Dietary surveys are needed to analyze if women in these countries are getting more exposed to dietary aflatoxins compared to men and the risks should be assessed accordingly.

APPENDICES

APPENDIX A: Effects of Aflatoxins on the Immune System: Evidence from Human and Mammalian Animal Research

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Abstract

Shortly after its discovery in 1960 aflatoxin – a fungal toxin or mycotoxin produced by the fungi *Aspergillus flavus* and *A. parasiticus* in food crops such as maize, peanuts and tree nuts – was found to cause liver cancer in humans and multiple animal species. Hence, regulations on maximum allowable aflatoxin levels in food around the world have focused on protecting humans from aflatoxin's carcinogenic effects. However, aflatoxin may also have non-carcinogenic health effects (e.g., immune toxicity) that are particularly relevant today. Our current review highlights the growing evidence that aflatoxin exposure adversely affects the immune system. Here, we critically evaluted the epidemiological and mammalian animal studies that link aflatoxin exposure with adverse effects on the immune system. We analyzed the studies by animal models used to test as well as by the effects on adaptive vs. innate immune functions. There is strong evidence that aflatoxin exhibits immunotoxicity that may compromise the ability of both humans and animals to resist infections. However, we found that the effects of aflatoxin on immune markers are inconsistent in the existing literature. Consequently, the extent of the immunotoxic effects of aflatoxin must be urgently clarified so that the contribution of such immunotoxicity to the overall burden of human infectious diseases can be established.

Key words: Aflatoxin, immune system, immunotoxicity, vaccination

1. Introduction

In many parts of the developing world, especially Sub-Saharan Africa, populations face co-exposure to dietary aflatoxins and multiple infectious agents (Mupunga, Mngqawa & Katerere 2017; Liverpool-Tasie *et al.*,2019). Hence, it is critically important to understand how aflatoxin exposure may affect immunity to infectious diseases in these at-risk populations. Aflatoxins are secondary fungal metabolites of *Aspergillus flavus* and *A. parasiticus*. In warm climates, the fungi frequently contaminate food and feed commodities such as maize, peanuts, tree nuts, spices, and cottonseed (Wu, Groopman & Pestka 2014; Alshannaq and Yu 2017). Dietary exposure to aflatoxins is more common in tropical and subtropical climates because the growth of *Aspergillus* is promoted by high temperatures, humidity and cycles of drought followed by heavy rainfall (Wagacha and Muthomi 2008). There are four major types of aflatoxins present in food crops: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). AFB_1 is the most toxic derivative and also the form most commonly found in food. Its hydroxylated metabolite aflatoxin M_1 (AFM_1) can be found in milk and other dairy products from dairy animals that have consumed AFB_1-contaminated feed. Therefore, vertical transmission of aflatoxin from mothers to infants via breast milk, as well as dairy products can potentially impact resistance to infections among infants and children.

In particular, aflatoxin exposure is a concern for populations in tropical and subtropical nations where maize and peanuts are dietary staples; such as in Sub-Saharan Africa, where mortality rates due to infectious diseases is very high (Vuuren 2017; WHO 2018b). Furthermore, in numerous studies, aflatoxin exposure has been associated with childhood stunting; which is considered a potential risk factor for immunological alterations. The literature about aflatoxin and growth impairment has already been covered in multiple review papers (Khlangwiset, Shephard

and Wu 2011; Mupunga *et al.*,2017; Watson, Gong & Routledge 2017); therefore, we will not include those studies in this manuscript.

Over the last 60 years, aflatoxin exposure has been associated with multiple adverse health outcomes. The US Food and Drug Administration (US FDA) has set an aflatoxin limit of 20 μg/kg for foods and also for most animal feeds (US FDA 2000). A much stricter limit for aflatoxin is enacted by the European Union (EU): 2 μg/kg AFB1 and 4 μg/kg total aflatoxins for nuts and cereals for human consumption (European Commission 2010). Aflatoxin exposure in food is considered a significant risk factor for liver cancer (Wild and Gong 2010). Consumption at high doses is associated with acute aflatoxicosis, acute liver damage, edema, and even death (Azziz-Baumgartner *et al.*,2005; Strosnider *et al.*,2006). It is suspected that consumption of food contaminated with 1 mg/kg or higher levels of aflatoxin may lead to aflatoxicosis (WHO 2018a). It was estimated from previous aflatoxin outbreaks that, consumption of 20–120 μg/kg (body weight) BW/day of AFB_1 within a period of one to three weeks is associated with acute toxicity and potential lethality (WHO 2018a). The International Agency for Research on Cancer (IARC) has classified "naturally occurring mixture of aflatoxins" as a Group 1 human carcinogen (IARC 2002). In fact, the risk of aflatoxin-related liver cancer is roughly thirty-fold higher for individuals who are simultaneously infected with hepatitis B virus (HBV) due to a possible synergistic interaction between HBV infection and the mutagenic capacity of aflatoxin (JECFA 1998; Moudgil *et al.*,2013; Wu, Stacey & Kensler 2013). The carcinogenicity of aflatoxins is related to the ability of their metabolites to interact with DNA. AFB_1 is metabolized in the liver by cytochrome P450 enzymes which leads to production of AFB_1 – 8-9 epoxide which is a very reactive metabolite (Kensler *et al.*,2011; Kew *et al.*,2013). This AFB_1 – 8-9 epoxide metabolite is highly unstable and binds to guanine bases in DNA to produce aflatoxin-N7-guanine adduct which

has a critical role to play in aflatoxin-induced genotoxicity. Furthermore, exposure to aflatoxin is also associated with growth impairment in children, pregnancy loss, premature birth, and immunotoxicity (Bondy & Pestka 2000; Gong *et al.*,2004; Khlangwiset *et al.*,2011; Wild, Miller & Groopman 2015; Smith *et al.*,2017; Watson *et al.*,2018; Lauer *et al.*,2019). Multiple studies conducted using animal and cell culture models have indicated that aflatoxin has immunotoxic effects (Bondy and Pestka 2002). However, the mechanisms by which aflatoxins result in immunomodulating effects have not been clearly determined. Previous studies have reported the reactive –8-9 epoxide to be potentially responsible for aflatoxin-induced immunomodulation. For instance, the –8-9 epoxide metabolite can interrupt DNA-dependent RNA polymerase activity which can inhibit synthesis of RNA and proteins (Raney *et al.*,1993). The –8-9 epoxide metabolite binding to DNA and interrupting protein synthesis might directly or indirectly affect the proliferation/differentiation of immune cells and interleukin production and therefore disrupt the communication between immune system mediators affecting both innate and adaptive immunity (Dugyala & Sharma 1996, Benkerroum 2020).

Because many readers are not immunologists, a brief introduction to the immune system and its major components is required. Readers who desire more detailed information about immunity are directed to review an introductory immunology textbook such as Janeway or Abbas (Abbas *et al.*,2017; Murphy & Weaver 2018). The immune system has two interacting components: the innate immune system and the adaptive immune systemm. The innate immune system is the first line of defense against infection, and the innate immune response is non-specific or broadly specific and provides a general type of protection against infections. It is crucial during the early minutes to hours of exposure to an antigen; for example, through a cut or scrape on the skin. Macrophages, dendritic cells, neutrophils, monocytes, basophils, eosinophils, mast cells, innate

lympoid cells and natural killer (NK) cells are the cellular components of the innate immune system. The adaptive immune system is dependent upon the the innate immune system to elicit a response. Its response (e.g. antibody production) is highly specific, tailored to protect against a specific infectious agent. Adaptive immunity takes 1-2 weeks to develop after expsoure to infection, and it can eliminate infections more efficiently than the innate system alone (Murphy and Weaver 2018). The two cellular components of the adaprive immune system are the bone marrow-derived, but thymus-differentiated lymphocutes (commonly called T cells) and the bone marrow-derived and also bone marrow-differentiated lymphocytes (commonly called B cells). T-cells are of the following major types---$CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells include T "helper" (Th) cells and T-regulatory cells. The Th cells play a crucial role in the adaptive immune system by helping B-cells to produce antibodies. The $CD8^+$ T cells are cytotoxic lymphocytes that are crucial for protection against viruses and tumors. It is noteworthy that vaccination programs against infections diseases are intended to elicit not only innater response, but also more importantly, adaptive immune responses together with a memory compoenent in the adaptive immune system to protect from infections. Thus, any toxic effects of aflatoxin on the innate or the adaptive immune cells will be expected to impair host immunity against infections.

Immune cells of both the innate and the adaptive system secrete signalling proteins called cytokines and chemokines, which activate target tissues and immune cells to enable more efficient immune responses against invading microbes through amplification of immune signaling (Murphy and Weaver 2018). Cytokines are often designated as either pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines include interleukin (IL)-1 α, ß, IL-2, IL-4, IL-6, IL-17, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ; and anti-inflammatory cytokines include IL-10, and transforming growth factor (TGF)-ß.

The primary objective of this research was to provide a critical review of the human studies and mammalian animal studies that have associated aflatoxins with immunotoxicity. We describe the effects of aflatoxin on the innate and the adaptive immune systems of humans and animals in separate sections. We also discuss the potential mechanisms by which aflatoxin may compromise the immune system and identify research gaps to provide direction for future research. We performed a PubMed and Google scholar database search using a combination of subject headings and free text words including: aflatoxin and immune system, aflatoxin and immune suppression, aflatoxin on T-cells, aflatoxin and vaccination. The step-by-step process of our literature search is presented in **Figure 10**. The search included all papers published between January 1980 to January 2021. Additionally, we screened the reference lists of the included studies to identify additional references relevant to our topic. We excluded *in vitro* cell culture studies because although such studies are useufl to understand mechanisms, it is very difficult to traslate the results from such studies to human health. We also excluded non-mammalian (chicken, duck, fish etc.,) *in vivo* studies as their immune systems are significantly different from that of humans). The search identified 27 articles (eight human studies and 19 animal studies) that matched the search criteria. These were analyzed to derive the syntheisized data presented that were used in interpretations.

2. Human studies

We identified and analyzed eight human studies that reported the association between aflatoxin exposure and markers of immune system function. Table 16 contains a detailed list of these studies including the number of participants for each study, the immune biomarkers analyzed and the results observed. We have summarized these studies below:

Effects of aflatoxin on the innate immune system. Natural Killer (NK) cells are components of the innate immune system that kill virus infected cells and release immunoregulatory cytokines. Macrophages are one type of professional antigen presenting cells that the immune system uses to process the antigens and then present them to the adaptive immune cells (T-cells and B-cells). Aflatoxin exposure affects NK cells but not macrophage populations in humans. Jiang *et al., (*2005) examined the relationship between the number of macrophages and NK cells and the levels of aflatoxin B_1 albumin adducts (AF-alb) in the plasma of Ghanaians (n=64). They quantified the numbers using specific cell markers as follows: macrophages, $CD14^+$; NK cells, $CD3^-CD56^+$; and subtypes of NK cells, $CD3^-CD56^{bright}CD16^{dim}$ and $CD3^-CD56^{dim}CD16^{bright}$. Based on AF levels they classified subjects into high (>0.9068 pmol mg−1 albumin) and low AF group (<0.9068 pmol mg−1 albumin). Participants with higher AF-alb had a slightly higher percentage of $CD3^-CD56^+$ NK cells compared to participants with lower AF-alb but the difference was not significant (4.24 vs 3.90; P, n.s.). The percentage of CD14+ macrophages was also similar in the two groups (). The high-AFB_1 group had a lower percentage of $CD3^-CD56^{bright}CD16^{dim}$ cells, which play a role in antibody-dependent cellular cytotoxicity (Poli *et al.*,2009), than the low-AFB_1 group but the difference was also not statistically significant (20.76 vs 27.12; P, ns.) (Jiang *et al.*,2005). This was the only human study we found that tested effects of AF on human innate cells.

Effects of aflatoxin on the adaptive immune system. The T cell and the B cell activation marker CD69 is an important regulator of immune responses and is important for activation of cytokine production and for T-helper cell differentiation (Cibrián and Sánchez-Madrid 2017). In the aforementioned Ghanaian cohort, Jiang *et al., (*2005) found the mean percentages of CD69 activation markers: $CD3^+CD69^+$ (T cells) and $CD19^+CD69^+$ (B cells) to be significantly lower in individuals who had higher levels of AF-alb. In these individuals with high AF-alb levels,

significantly lower percentages of perforin-expressing and perforin- and granzyme A- expressing $CD8^+$ T-cells were observed (Jiang *et al.*,2005). These CD8+ T cells are cytotoxic killer cells that play a critical role in protection and recovery from intracellular pathogens such as viruses and protozoan parasites by killing the pathgoen infected cells and thereby inhibiting the spread of pathogens (Liu, Walsh and Young 1995). Thus, these results suggest that high AFB_1 levels impair $CD8^+$ T-cell function thereby compromise host defense against such pathogens. Other lymphocyte subsets, such as CD3+ T-cells, $CD4^+$ T cells, $CD8^+$ T cells, CD19+ B-cells and IFN-γ- and IL-4-expressing $CD4^+$ T cells, did not differ between the low vs. high AF-alb groups.

High aflatoxin exposure may encourage more rapid human immunodeficiency virus (HIV-1) disease progression in HIV-infected people, which was demonstrated by Jiang *et al., (*2008). This study analyzed multiple immune parameters to investigate the interaction of aflatoxin and HIV-1 on immune system impairment in HIV-1 positive (n=161) and HIV-1 negative (n=80) Ghanaians. In both groups, higher levels of AF-alb were associated with lower levels of $CD4^+$ T-regulatory cells and naïve $CD4^+$ T-cells. Similar to their previous study results (Jiang *et al.*,2005), higher plasma levels of the AF-alb were associated with lower expression of perforin in $CD8^+$ T-cells. HIV-1 positive patients with high AF-alb levels also had a significantly decreased percentage of B cells. These results indicate that high aflatoxin exposure may facilitate rapid progression of HIV-1 disease by reducing the number and function of T helper cells, T-regulatory cells, CD8+ T cells and B cells (Jiang *et al.*,2008).

In another Ghanaian cohort, the plasma AF-alb levels of HIV-1 negative (n=159) and positive (n=155) participants were measured and the differences in clinical factors, including $CD4^+$ cell count, antibody to HBV surface antigen (HBsAg), Hepatitis C virus (HCV) antigen in plasma and *Plasmodium falciparium* antigen were examined (Jolly *et al.*,2011). Significantly higher AF-alb

levels were observed in the plasma of HIV-infected participants compared with that of HIV-uninfected participants indicating aflatoxin exposure may contribute to higher viral loads. However, in HIV-infected participants, $CD4^+$ T-cell counts did not differ on the basis of plasma AF-alb. Therefore, aflatoxin exposure had no significant effects on $CD4^+$ T-cells. In a following study, Jolly *et al., (*2013) examined the association between aflatoxin exposure and HIV-1 viral load in antiretroviral therapy (ART) naïve, HIV-positive adults (n=314) with median CD4+ T cell counts of 574 cells/µl blood in Ghana. They found significantly higher viral loads in HIV-positive individuals who had higher AF-alb levels in their blood (Jolly *et al.*,2013). The results of this Ghanaian study also imply that the immune modulatory effects of aflatoxin occur even before the $CD4^+$ cell count decreases below 500/µl blood. The authors concluded that aflatoxin and HIV may have a synergistic effect on the immune system impairment resulting in higher viral loads early in HIV infection. Even though the abovementioned studies by Jiang *et al.*,2008, Jolly *et al.*,2011 and Jolly *et al.*,2013 demonstrate that HIV-infected patients tend to have higher AF-alb levels in the plasma than non-infected individuals, this difference could be due to aflatoxin inducing higher viral loads, or due to socioeconomic differences; HIV-infected individuals may have higher exposures to lower quality or moldy maize resulting in increased exposures to dietary aflatoxins (Williams *et al.*,2005).

Secretory immunoglobulin A (sIgA), which is an important component of the mucosal barrier that binds to bacterial and viral surface antigens, has a negative correlation with aflatoxin exposure. In a study of 472 Gambian children, the authors investigated the effect of dietary aflatoxin exposure on sIgA in saliva and cell-mediated immunity (CMI), and antibody responses to rabies, and pneumococcal vaccine (Turner *et al.*,2003). Levels of sIgA were significantly lower in children with detectable serum AF-alb compared to those with nondetectable levels. Antibody

response to one of the pneumococcal serotypes (serotype 23) was positively but weakly associated with higher levels of AF-alb but the rabies antibody titers and the other pneumococcal serotype antibody titers were not associated with AF-alb (P > 0.05) (Turner *et al.*,2003). Allen *et al., (*1992) investigated the association between serum AF-alb levels and immunological features of malaria and HBV infection in 391 Gambian children. They found AF-alb levels to be higher in children who were positive for Hepatitis B surface antigen (HbsAg) and *Plasmodium falciparum* parasitaemia compared to controls.

Aflatoxin exposure might be associated with Hepatitis B surface antigen antibody (anti-HBs) levels, which was demonstrated by a recent study investigating the immune modulation effects of dietary aflatoxin exposure in Kenyan children aged between one and fourteen years by studying the anti-HBs antibody levels (Githang'a *et al.*,2019b). Only 47.8% (98 out of 205 children) of those Kenyan children tested positive for anti-HBs antibody even with the high coverage of routine immunization. The results of this study indicated that for every unit rise in AF-alb level in serum, the level of anti-HBs antibody decreased by 0.91 mIU/ml, indicating a weak association (P = 0.19) between exposure to aflatoxin and antibody response. However, there is a possibility of reverse causation, that is, presence of HBV may decrease the inactivation of AFB-epoxide, leading to greater production of AF-alb in serum. This study also analyzed serum IL-2, IL- 4, IL-6, IL-8, IL-10, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-γ levels. AF-alb levels were negatively correlated with all cytokines except IL-10, TNF-α and GM-CSF. However, none of these associations were statistically significant (Githang'a *et al.*,2019b)

Interleukin 10 (IL-10) is a cytokine that has anti-inflammatory properties and plays a critical role in limiting immune response to pathogens and maintaining normal tissue homeostasis (Iyer and Cheng 2012). A recent case study determined the possible association between IL-10 in cord

blood and patients with gestational diabetes (GD) who are exposed to aflatoxin (Xie *et al.*,2018). The results indicated that the IL-10 levels in cord blood samples of AFB_1 exposed GD patients were significantly higher compared to non-GD controls. The study concluded that IL-10 may serve as a biomarker for immunoregulation in GD patients exposed to aflatoxin (Xie *et al.*,2018). However, this study had a very small population size (n=3 per group) so the results need to be confirmed in future studies.

Taken together, the association between aflatoxin exposure with alterations in human immune system markers is not conclusive, considering some of these studies were cross-sectional, had very small population size and have not been confirmed. However, since two of the studies have linked aflatoxin exposure with possible adverse disease outcomes, such as more rapid progression of HIV (Jiang *et al.*,2008) and impaired vaccine response (Turner *et al.*,2003), the potential immunotoxic impact of aflatoxin in humans needs to be ascertained.

3. Experimental animal studies

The adverse effects of aflatoxin exposure on various markers of the immune system have been demonstrated in multiple animal species over the last few decades (reviewed in Bondy and Pestka 2000; Mohsenzadeh *et al.*,2016). Here, we have summarized the mammalian *in vivo* studies that looked at effects of aflatoxin on immune system markers, describing the results among different species and, innate and adaptive immune responses. The study details including species of animal, doses of aflatoxin used, the immune biomarkers analyzed and the results are listed on Table 17.

Mice

Effects of aflatoxin on the innate immune system. Low dose of AFB_1 exposure (30 µg/kg BW/every other day for two weeks) can significantly decrease white blood cell (WBC) counts. This was

consistently observed in three different studies which included two different mice strains (CD-1 and Balb/c) (Reddy *et al*., 1987; Reddy and Sharma, 1989; Dugyala and Sharma 1996). Neutrophils and monocytes are two important innate immune cells that might be affected by aflatoxin exposure. A dose-dependent suppression in NK cell-mediated cytolysis of YAC-1, a lymphoma cell line, was found using NK cells from mice treated with 30, 145 or 700 µg AFB_1/kg BW orally (gavage in corn oil) every other day for four weeks (Reddy and Sharma 1989). Mice orally treated with 200 µg/kg BW/day AFB_1 for 24 days also experienced significant decreases in the number of neutrophils and monocytes in the blood compared to the control group (Tomková *et al.*,2002). In contrary, Tuzcu *et al., (*2010) found significantly higher proportions of neutrophils (increased in a dose-dependent manner) and no significant change in monocyte proportions in the peripheral blood of mice treated with aflatoxin (up to 1600 ppb, ≈ 300 µg/kg BW/day) compared to the control. The contradictions in the results could be due to difference in treatment durations (Tuzcu *et al.*,2010 did not mention the specific mice strain used and duration of treatment). Aflatoxin exposure may also affect the proportions of peripheral blood eosinophils which play important role in defense against viral, parasitic and bacterial infection (Wen 2017). Tuzcu *et al., (*2010) observed a significant decrease in peripheral blood eosinophil levels in aflatoxin-treated groups compared to the control. However, there was no significant change in proportions of basophils except in the mice receiving the lowest dose of aflatoxin (200 ppb ≈ 40 µg/kg BW/day) which showed a significant decrease. Aflatoxin exposure may have an effect on the systemic immune response in mice infected with *Encephalitozoon cuniculi* (Levkutová *et al.*,2003). In this study, mice were distributed into four groups and orally administered– control, AFB_1, E. *cuniculi* or AFB_1 + E. *cuniculi* for 27 days. At 27 days of post-treatment, the AFB_1- treated mice showed a significant reduction in leukocyte and neutrophil counts compared to the control group. AFB_1

exposure in mice infected with E. *cuniculi* also resulted in significant quantitative increase in monocytes compared to the control group (Levkutová *et al.*,2003).

Aflatoxin exposure was associated with a decrease in phagocytosis and the production of macrophage metabolites [nitric oxide (NO), hydrogen peroxide (H_2O_2, superoxide anion (O_2^-)] and also altered cytokine production by macrophages (Dugyala and Sharma 1996; Moon *et al.*,1999b). TNF-α cytokine production by macrophages was reduced in mice exposed to 400 µg AFB_1/kg BW every other day for 2 weeks AFB_1 (Moon *et al.*,1999b). On the other hand, Dugyala and Sharma (1996) found significant increase in the mRNA levels of TNF-α produced by macrophages starting from the medium dose of AFB_1 (145 µg/kg BW every other day) even though both these studies used the same mice strain (CD-1) and treatment durations. Low dose of AFB_1 (30 µg/kg BW every other day) significantly increased mRNA levels of IL-1α produced by macrophages and IL-6 at the medium dose (145 µg/kg BW every other day) (Dugyala and Sharma 1996). However, high-dose of AFB_1 (700 µg/kg BW every other day) showed a significant reduction of both IL-l-α and TNF-α produced by macrophages in mice (Dugyala and Sharma 1996). Therefore, the change in cytokine levels produced by macrophages depends on the dose of AFB_1. Exposure to AFG_1, another type of aflatoxin, also may also have an effect on macrophage production (Liu *et al.*,2015). An oral administration of 100 µg/kg BW/day AFG_1 for one month resulted in an increase in alveolar $CD68^+$ macrophages which peaked after three and six months of aflatoxin exposure (Liu *et al.*,2015).

The effect of AFM_1, a metabolite of AFB_1 found in milk, was investigated on various aspects of innate immunity including white blood cells (WBC) counts, phagocytic capacities of monocytes and granulocytes by Srirani *et al., (*2018). However, no significant differences in numbers of total WBC, monocytes or neutrophils nor in the phagocytic capacities of monocytes or granulocytes

were observed in the mice receiving 25 and 50 μg/kg BW/day AFM_1 for 5 days a week for a total of 4 weeks (Shirani *et al.*,2018).

The studies mentioned above indicate, aflatoxin exposure in mice affects either proliferation on function of many components of the innate immune system including neutrophils, eosinophils, basophils, monocytes, NK-cells, macrophages and cytokines produced by macrophages. However, the effects are not consistently observed in all studies which could be due to the difference in strains of mice used, dose, duration of exposure and route of exposure.

Effects of aflatoxin on the adaptive immune system. AFB_1 treatment can significantly affect the lymphocyte proportions and the percentages of alpha naphthyl acetate esterase (ANAE) positive peripheral blood lymphocytes (T-lymphocytes), which play important roles in endocytosis and degradation of antigens and cytotoxic effects of activated T-cells (Tuzcu *et al.*,2010). Both the proportions of peripheral blood lymphocytes and the proportions of ANAE-positive peripheral blood lymphocytes decreased significantly in the aflatoxin-treated groups compared to the control group in a dose-dependent manner (Tuzcu *et al.*,2010). Significant decreases in the lymphocyte counts and the proportion of $CD3^+$T-cells in the intestinal mucosa was observed in AFB_1 (200 µg/kg BW/day) treated mice as compared to the control group after 24 days of exposure (Tomková *et al.*,2002). Similar reductions in the proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes were also observed at a much higher dose of AFB_1 exposure (750 µg/kg BW/day through intragastric administration for 30 days) in mice (Xu *et al.*,2019). AFM_1, a metabolite of AFB_1 found in milk, has also been shown to reduce $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cell percentages in the spleens of exposed-mice compared to non-exposed (Shirani *et al.*,2018). AFG_1-exposed mice indicated an increase in $CD3^+$ lymphocytes in the alveolar septum starting at one month, which peaked at three and six months of AFG_1 treatment (Liu *et al.*,2015).

Production and mRNA expressions of cytokine, chemokine and transcription factors by adaptive immune cells are also altered by aflatoxin exposure. A decrease in mRNA expression levels of lymphocytic cytokines IL-2, IFN-γ, and IL-3 was observed at a lower dose (30 µg AFB_1/kg BW/every other day); however, the difference was only significant for IL-2 ($P < 0.05$) (Dugyala and Sharma 1996). Significant reductions in the contents of IL-2, IFN-γ and TNF-α in serum and IL-2, IFN-γ and TNF-α mRNA expression in spleen were observed in mice receiving a high dose (750 µg AFB_1 /kg BW/day for two weeks) compared to the control group (Xu *et al.*,2019). On the other hand, a single AFB_1 dose (663 µg AFB_1/kg BW/day) induced upregulation of IL-4 and IFN-γ cytokines expressions in liver and there was no significant change observed between the IL-17 cytokine expression in the livers of aflatoxin treated and untreated mice groups (Ishikawa *et al.*,2017). In terms of AFM_1 exposure, it did not have a significant effect on IL-4 levels, but it significantly decreased IFN-γ and increased in IL-10 levels (Shirani *et al.*,2018). Mice treated with AFG_1 showed an increase in TNF-α, IL-1β, IL-6 expressions at each time point (100 µg/kg BW at one, three and six months) following AFG_1 gavage (Liu *et al.*,2015). AFG_1 treatment also increased expressions of chemokines (CCL-2, CXCL-2 and CXCL-1), which are important mediators in a chronically inflamed microenvironment of the lungs of mice (Liu *et al.*,2015). This study also found an up-regulation of NF-κB, p-STAT3 and COX 2 expressions in alveolar epithelial cells (Liu *et al.*,2015). There were no other mice studies identified on effects of aflatoxins on chemokines and transcription factors. The inconsistent findings on cytokine levels following aflatoxin exposure imply that, the changes in the cytokine levels caused by aflatoxin depend on the duration of the exposure.

AFB_1 can significantly inhibit the number of IgM class antibody-producing cells in spleen against sheep red blood cells (Reddy *et al.*,1987). However, it did not have any effect on the

number of T-independent antibody-producing cells. AFM_1 also showed to significantly decrease IgG concentrations in the blood serum of exposed-mice but did not affect the concentrations of IgM (Shirani *et al.*,2018).

A single oral dose of 442 and 663 µg AFB_1/kg BW significantly suppressed the proliferative response for Con-A-stimulated lymphocytes (polyclonally activated T-cells by Con-A lectin) (Ishikawa *et al.*,2017). AFB_1 exposure can also suppress delayed-type hypersensitivity response to keyhole limpet hemocyanin (KLH) in mice (Reddy *et al.*,1987).

As a consequence of difference in the experimental designs, the results of the mice studies analyzing the effects of aflatoxin exposure on the adaptive immune system components are conflicting, in terms of T-cell subsets and cytokine production and expression levels. However, there is cogent evidence from the mice studies that aflatoxin is able to alter components of adaptive immune system which can potentially affect both cellular and humoral immunity.

Rats

Effects of aflatoxin on the innate immune system. Rats treated with 200 AFB_1/kg feed (≈ 30 µg AFB_1/kg BW/day for 8 weeks) showed significant reduction in total WBC counts including lymphocytes and monocytes, significant increase in neutrophil count, and no change in eosinophils and basophils counts (Essa *et al.*,2017). This study also observed significant reduction in the phagocytic activities by both neutrophils and macrophages in AFB_1-exposed group compared to the control. A higher dose of AFB_1 exposure (300 µg/kg BW) also caused reduction in phagocytic function (by 50% compared to control) (Raisuddin *et al.*,1994). On the other hand, an increase in the total WBC count in whole blood was observed in Fisher-344 male rats continuously treated with 1600 µg AFB_1/kg diet ≈ 1032 µg AFB_1/kg BW/day (assumed average BW of rats is 31g and

the rats were fed 20g feed per day) for 8 weeks (Hinton *et al.*,2003) These contradictory effects on WBC and neutrophils might be due to the large difference in AFB_1 doses tested. The rats in the Hinton *et al* (2003) study also showed a significant decrease in the percentage of segmented neutrophils only after 12 weeks of treatment at ≈ 1032 µg AFB1/kg BW/day; the lower doses did not indicate any significant changes in WBC counts and percentage of segmented neutrophils at other time points (4, 8, 16 and 20 weeks).

AFB_1 exposure for five days a week indicated a dose-dependent decreases in the percentage of CD3-CD8a+NK cells in rats compared to the control animals after just one week of AFB_1 treatment (Qian *et al.*,2014). After five weeks of AFB_1 treatment, an increase in the percentage of TNF-α expression by NK cells was observed in the highest dose group (75 µg/kg BW) which may contribute to chronic inflammation (Qian *et al.*,2014). AFB_1 exposure orally for two weeks on alternate days in rats indicated suppression in delayed type of hypersensitivity response in terms of foot pad thickness (Raisuddin *et al., (*1994).

The limited studies regarding effects of aflatoxin on the innate immune system of rats indicate alteration in percentage of innate immune cells including monocytes, neutrophils, NK cells, percentage of TNF-α expression by NK cells, WBC counts in blood and phagocytic function.

Effects of aflatoxin on the adaptive immune system. There is evidence that aflatoxin exposure can suppress evels of both T-cells (including different T-cell subsets) and B-cells in rats. Treatment with 300 µg AFB1/kg BW orally for two weeks on alternate days significantly reduced cell counts of thymus and bone marrow in rats (Raisuddin *et al.*,1994). This study also found that the peritoneal exudate immune cell population in AFB_1-exposed rats is severely depleted (40%) compared to the control animals. AFB_1 treatment caused significant depression in mitogenesis of T- and B-cells in exposed-rats compared to control animals (Raisuddin *et al.*,1994). The T-cell and

B-cell percentages in the spleens of exposed rats were affected after intermittent exposure of AFB_1 (≈ 258 µg AFB1/kg BW/day for 4 weeks on and 4 weeks off for 20 weeks) (Hinton *et al.*,2003). In this study, the percentages appeared to either reverse or compensate after the off cycles; after 12 weeks, the T-cell percentage significantly increased while the B-cell percentage significantly decreased, but after the off-cycle (at 16 weeks), the T-cell percentage decreased while the B-cell percentage increased (Hinton *et al.*,2003). At intermittent exposure to this dose, this study also found significant increase in the percentage of CD4+ T-cell subset at 8 weeks and no significant change at other time points. However, the percentage of CD8+ T-cell subset increased at 12 weeks, decreased at 16 weeks and increased back at 20 weeks suggesting a compensatory change in response after different off-cycles (Hinton *et al.*,2003). Qian *et al., (*2013) found a dose-dependent decreases in the percentage of splenic $CD8^+$ T cells in rats treated with 5–75 µg/kg BW for one week. This study also analyzed the effects of 5-week of AFB_1 exposure and found an increase in the percentages of $CD3^+$ and $CD8^+$ T-cells in the animals exposed to low doses (5 and 25 µg AFB_1/kg BW for 5 days a week). However, there was no significant change observed in $CD4^+$T-cells and B-cells after 5 weeks of treatment (Qian *et al.*,2014).

Rats orally exposed to 1000 µg AFB_1/kg BW/week for five consecutive weeks with ovalbumin (OVA) showed an increased number of $CD8^+$ and $CD8/CD71^+$ cells in mesenteric lymph nodes indicating activation of T-suppressor cells, however, same effect was not observed in rats exposed to a low dose (100 µg AFB_1/kg BW/week) (Watzl *et al.*,1999). In this study, neither of the AFB_1 doses showed any effect on the ratio of $CD4^+/CD8^+$ lymphocytes, the percentage of $CD4^+$ and $CD8^+$ lymphocytes in mesenteric lymph nodes and in the spleen, and in the serum concentrations of OVA specific IgE and IgG antibodies (Watzl *et al.*,1999).

Based on the exposure window of dose and time, the effects of AFB_1 on the immune system can either be stimulatory or suppressive (Hinton *et al.*,2003). After stimulation with lipopolysaccharide (LPS) or LPS and IFN-γ, Hinton *et al* (2003) analyzed the effects of intermittent exposure of AFB_1 on inflammatory response by measuring IL-1, IL-2 and IL-6 levels at different time points with on and off cycles of exposure to AFB_1. The results did not indicate any consistent pattern in the cytokine levels with on and off exposures and time but the significant increase in both IL-1 and IL-6 at 12 weeks suggested induction of inflammatory response (Hinton *et al.*,2003). Qian *et al., (*2013) also found evidence that AFB_1 exposure may promote inflammatory responses after repeated exposures. In this study, rats exposed to 25 µg/kg BW for 5 days a week showed significant increase in the percentage of proinflammatory IFN-γ expression but a decrease in the IL-4 expression by $CD4^+$T-cells.

Similar to the mice studies, the effects of aflatoxin on the adaptive immune system of rats are also inconsistent. In some studies, the T-cell subsets increased following aflatoxin exposure, while in some, it decreased. Similar inconsistencies were observed for cytokine levels as well. The effects on antibody production were analyzed by only one rat study (Watzl *et al.*,1999), which was not affected by aflatoxin; perhaps a higher dose regimen of AFB_1 (more than once a week) was required to observe a difference. The experimental designs in the above-mentioned rat studies are very different which might explain the contradictions in the findings. Nonetheless, the studies still provide strong evidence that aflatoxin can affect adaptive immune system components leading to immunomodulation.

Pigs

Effects of aflatoxin on the innate immune system. AFB_1 exposure shows inconsistent effects in WBC counts in pigs. Three-weeks old pigs exposed to 344 μg AFB_1/kg BW/day had an increased WBC counts in blood, particularly neutrophils, compared to the controls. The monocyte count was not significantly different in the exposed-pigs compared to the control animals (Meissonier *et al.*,2008). However, in another study, no significant change was observed in the relative number of neutrophils, monocytes, basophils, and eosinophils in blood of four-weeks old piglets fed with low doses of AFB_1 (≈30 and 60 μg AFB_1/kg BW/day) in feed for 30 days (Marin *et al.*,2002).

The limited number of pig studies looking at the effects of aflatoxin on innate immunity of pigs did not find any major alteration in the innate immune components following aflatoxin exposure except an increase in the WBC counts (especially neutrophils) observed when pigs were exposed to a high dose (≈ 344 μg AFB_1/kg BW/day) by Meisonnier *et al., (*2008).

Effects of aflatoxin on the adaptive immune system. AFB_1 exposure in pigs indicate incoherent effects on cytokine production and mRNA expression levels. Pigs consuming 344 μg AFB_1/kg BW/day in the feed showed significant increase in mRNA expression of TNF-α, IL-1β, IL-6, IFN-γ and IL-10 (Meissonier *et al.*,2008). In contrary to this study, Marin *et al., (*2002) found low doses of AFB_1 exposure (≈30 or 60 μg AFB_1/kg BW/day) to decrease the mRNA synthesis of IL-1β significantly and slightly decreased TNF-α, but it was not significant ($P > 0.05$). This study also found that aflatoxin exposure did not modify IL-2 and IL-4 production in pigs, but IL-10 mRNA synthesis was upregulated.

AFB_1 exposure in pigs did not result in any major change in antibody concentrations. Van Heugen *et al.*,1994, investigated antibody response to sheep red blood cells (SRBC) and to total serum IgM and IgG concentrations after feeding animals with ≈ 40 or 80 μg AFB_1/kg BW/day for

3 weeks. No differences were observed in antibody response to SRBC and serum IgM and IgG levels in either of the experimental groups compared to the control group (Van Heugen *et al.,*1994). Meissonier *et al., (*2008) analyzed the total concentration of IgA, IgG and IgM and anti-OVA IgG in plasma after stimulation with concanavalin A or OVA, but did not find any significant effect of AFB_1 exposure at any of the doses tested. Marin *et al* (2002) did observe a dose-dependent increase in the concentration of γ-globulin (contains antibodies) in the serum of AFB_1-exposed piglets, however AFB_1 had no effect on total globulin concentration in serum. This study also looked at the serum antibody levels after immunization with *M. agalactiae* and found it to be lower in aflatoxin-exposed groups but the differences were not significant ($P > 0.05$) compared to the control group (Marin *et al.,*2002). A dose-dependent impaired proliferation of lymphocytes during stimulation with OVA antigen was observed in pigs (Meissonier *et al.,*2008). The authors concluded that the delay and reduction in the lymphocyte proliferation could be associated with a reduced T-cell activation during the vaccination protocol (Meissonier *et al.,*2008).

All three studies found similar results in terms of effects of aflatoxin on antibody production which were not significant. Both Meissonier *et al., (*2008) and Marin *et al., (*2002) found increase in IL-10 levels following aflatoxin exposure, however, different effects were observed for TNF-α and IL-1β.

4. Discussion

In low-income nations, the majority of childhood deaths result from infectious disease. More than two million children die each year from diseases that are vaccine-preventable (Duclos *et al.,*2009; Gavi 2009; USAID). Sub-Saharan Africa has the highest under-5 mortality rate in the world: 14 times higher than the rate in high-income nations (WHO 2018b). Aflatoxin contamination of staple foods such as maize and peanuts is common throughout sub-Saharan Africa. This results in chronic

dietary exposure to aflatoxin in many populations (Xu, Gong & Routledge 2018). Our review indicates that there is strong evidence that aflatoxin exposure may increase the risk of immune system dysfunction by disruption of both innate and adaptive immunity and by decreasing the efficacy of vaccination. The limited epidemiological studies indicate that aflatoxin exposure is associated with impairments in both cellular and humoral immunity.

The mechanisms by which aflatoxin may cause immune dysfunction cannot be confirmed, due to differences in study designs and use of different animal species, but several possible mechanisms have been identified. Figure 11 summarizes the evidence of the different ways that aflatoxin leads to immonomodulation. Multiple studies have indicated that aflatoxin exposure can impair innate immune cells including macrophages, neutrophils and NK cell-mediated functions (Reddy and Sharma 1989; Neldon-Ortiz and Qureshi 1992; Silvotti *et al.*,1994; Cusumano *et al.*,1996; Bonomi & Cabassi 1997; Moon, Rhee & Pyo 1999a, Cheng *et al.*,2002; Meissonnier *et al.*,2008; Mohsenzadeh *et al.*,2016). Aflatoxin exposure was found to decrease T- and B-lymphocyte activities, which are the key cellular components of the adaptive immune response (Richard, Thurston & Pier 1978; Reddy, Taylor & Sharma 1987; Hinton *et al.*,2003; Jiang *et al.*,2015). There is evidence that indicate aflatoxin exposure can alter the levels of cytokines produced by both innate and adaptive immune cells (Hinton *et al.*,2003; Meissonnier *et al.*,2008; ; Li *et al.*,2014; Qian *et al.*,2014; Jiang *et al.*,2015; Ishikawa *et al.*,2017; Shirani *et al.*,2018; Wang *et al.*,2018).

The limited studies in humans that explored the effects of aflatoxin on the immune system have suggested impairments in cellular immunity (Turner *et al.*,2003; Jiang *et al.*,2005) and overall immunological response in humans. Controlling for other factors, high aflatoxin exposure was associated with more rapid HIV disease progression; possibly due to reduced $CD4^+$ and $CD8^+$ T-

cell counts in individuals who are already infected with HIV (Jiang *et al.*,2008). Studies have also indicated that aflatoxin may contribute to increases in HIV viral load which can impose greater risk of HIV transmission (Jolly *et al.*,2013; Jolly 2014).

Some animal studies indicated aflatoxin exposure can induce an inflammatory status and the impairment of the cellular immune response (Meissonnier *et al.*,2008). This might be associated with inhibitory effects of pro-inflammatory cytokines on the antigen presentation and antigen-specific immune response, resulting in ineffectiveness of vaccination protocols and also increase vulnerability to infections. However, other studies have found the opposite effect on inflammatory cytokines from aflatoxin exposure – suppression in pro-inflammatory cytokines following aflatoxin exposure (Moon *et al.*,1999b; Jiang *et al.*,2015; Shirani *et al.*,2018; Wang *et al.*,2018). Nonetheless, the evidence clearly shows how aflatoxin exposure alters cytokine expression and production levels. In addition to these contrasting effects on cytokine levels, we also observed different studies showing contradictory results on levels of subsets of T-lymphocytes associated with aflatoxin exposure. *In vivo* studies by Tomková *et al., (*2002), Jiang *et al., (*2015), Shirani *et al., (*2018), Wang *et al., (*2018)- all indicated that aflatoxin exposure is associated with suppression in T-cell subsets. On the other hand, studies by Hinton *et al., (*2003) and Kraieski *et al., (*2017) indicated T-cell lymphocytes to increase following exposure to aflatoxin. These variations in the results may occur due to using different routes of exposure, different doses and dosing regimens as stated previously in the review by Bondy and Pestka (2000). Although, the high doses used for most of these animal studies are irrelevant for most parts of the world where aflatoxin regulations are enforced (primarily in high-income countries and some middle-income countries), the doses are within the range of human exposure in some low income-countries where people may get exposed to very high levels of aflatoxin (up to 1400 µg/kg food) (Liverpool-Tasie *et al.*,2019).

Studies also suggest that even short exposure to lower levels of aflatoxin can also alter the immune response (Qian *et al.*,2014; Shirani *et al.*,2018). Additional experiments are required to determine if a dose threshold exists for aflatoxin to cause suppression or upregulation of cytokines and T-lymphocyte functions.

Based on our review on some animal studies, we found that aflatoxin has a suppressive effect on innate immunity (Tomková *et al.*,2002; Hinton *et al.*,2003; Levkutová *et al.*,2003; Tuzcu *et al.*,2010) and this is important in terms of many infections, for example, COVID-19 because the innate immune system act as the primary defense against viral infections (McKechnie and Blish 2020; Zhou *et al.*,2020). Eventhough the results have been contradictory, in some human and animal studies, we found evidence that aflatoxin appears to dampen the adaptive immune response which also play critical roles in protecting against infections and diseases.

It is estimated that around three million children die every year, mainly in low- and middle-income countries, from vaccine preventable infectious diseases (Duclos *et al.*,2009). Even though vaccination ranks among the most cost-effective tools in public health, the effectiveness of it can be influenced by many environmental factors, hence not all children around the world develop the same protective immune response to the same vaccine (Githang'a *et al.*,2019a; Githang'a *et al.*,2019b). There is evidence that aflatoxin can cross the placental barrier to the fetus and can also be excreted in breast milk (Khlangwiset *et al.*,2011; Smith *et al.*,2017). Therefore, exposure to aflatoxin can occur during critical developmental stages of the immune system. The studies mentioned above have indicated how aflatoxin may affect both innate and acquired immune responses which may also impact the effectiveness of vaccination. Some studies exploring effects of aflatoxin on effectiveness of vaccination have indicated that aflatoxin exposure does indeed impair vaccine response (Batra *et al.*,1991; Azzam and Gabal 1998; Meissonier *et al.*,2008; Yunus

and Böhm 2013); this means even if people receive vaccines in Sub-Saharan Africa or other high-risk areas with high exposure to dietary aflatoxins, their response to vaccines may be impaired. This is a particularly critical outcome; as in developing countries, vaccine-preventable infectious diseases are known to be a major cause of child mortality. Also, dietary aflatoxin exposure is more common in developing countries, which increases the likelihood of impaired vaccine responses in the vulnerable children in these populations. Nevertheless, not many studies have explored the effects of aflatoxin on vaccine responses, therefore, more studies should be conducted to confirm these results and to identify if these results are reproducible in other animal species and possibly in humans.

In summary, we encountered difficulties in conducting this review since we found many heterogeneities in the findings of the studies that we have reviewed, since the study designs are very different. Nonetheless, there is substantial evidence that aflatoxin exposure modulates diverse parts of the immune system. However, at the present moment, none of these results can be translated to a specific adverse health effect, as the immune system is extremely complex. Thus, it is difficult to comprehend the extent to which the immunomodulatory effects of aflatoxin affect the overall burden of human disease. This is an important area for future studies.

Table 16: Epidemiological studies of the effects of aflatoxin exposure on immune system markers.

Participants	Biomarkers analyzed	Results	Reference
A cohort of 64 Ghanaians (AF-alb range: 0.3325 to 2.2703 pmol/mg with a mean of 0.9972 +- 0.40 pmol/mg)	% of leukocyte immunophenotypes in peripheral blood, $CD4^+$ T cell proliferative response, $CD4^+$ Th and $CD8^+$ T cell cytokine profiles, NK cells (CD3-CD56+), macrophages (CD14+), and subtypes of NK cells: CD3-$CD56^{bright}CD16^{dim}$ and CD3-$CD56^{dim}CD16^{bright}$, monocyte phagocytic activity, NK cell cytotoxic function (perforin and TNF-α expression in $CD3^-$ $CD56^+$ NK cells)	• Strong negative correlations between the % of CD3+CD69+ cells (P = 0.001), and CD19+CD69+ cells (P = 0.032) and AFB_1 levels • High AFB_1 levels were significantly associated with lower % of CD3+ and CD19+ cells (P = 0.002) • No significant difference in $CD4^+$ T cell proliferative response • CD8+ T cells containing perforin and CD8+ cells containing both perforin and granzyme A were significantly lower in participants with high AFB_1 • No significant difference in monocyte phagocytic activity • High-AFB_1 group had a slightly higher % of NK cells and a lower % of CD3-$CD56^{bright}$ $CD16^{dim}$ cells (not significant)	Jiang *et al*., 2005

Table 16 (cont'd)

		• No significant difference in perforin and TNF-α expression in $CD3^{-}$ $CD56^{+}$ NK cells	
116 HIV+ and 80 HIV- subjects in Ghana AF-alb range: 0–3.48 pmoL/mg with mean level of 1.01 ± 0.53 and median of 0.91 pmoL/mg albumin	% of T-cells (CD3+), subsets of T-cells (CD4+ and CD8+), B-cells (CD19+), and NK-cells (CD3-CD56+), naive CD4 cells, CD8+ T-cell cytokine expression (perforin and granzyme A), cytotoxicity potential of NK-cells	• HIV positive patients who had high AF-alb had significantly lower % of CD4+ T regulatory cells (P = 0.009) and naive CD4+ T cells (P = 0.029) • Significant decrease in CD69 % on CD3+ T cells found in the high AF-ALB group among the HIV- controls • HIV + patients with high AF-alb levels had significantly lower % of B-cells (P = 0.03) compared to those with low AF-alb levels • High AF-alb levels were associated with lower expression of perforin on CD8+ T cells (P = 0.012) • CD8+ T cells containing both perforin and granzyme A were significantly higher in HIV + patients with high AF-alb (P = .000) and low AF-alb (P ≤ .003) compared to HIV- controls	Jiang *et al*., 2008

Table 16 (cont'd)

		• No significant difference in cytotoxicity potential of NK-cells	
Cross-sectional study of 314 (155 HIV +, 159 HIV -)	CD4 cell count, HBsAg, HCV antigen in plasma and *Plasmodium falciparium* antigen	• Significantly higher AF-alb levels was observed in HIV-infected participants (1.06 ± 0.60 pmol/mg albumin) compared to HIV-uninfected participants (0.91 ± 0.46 pmol/mg albumin). • Difference in CD4+ T-cell counts was not statistically significant between HIV positive participants with high and low aflatoxin exposures • No significant difference was observed in HIV-positive and -negative participants in terms of HBV and HCV infection and malaria parasitaemia	Jolly *et al.*, 2011
Cross-sectional study with 314 ART naive HIV+ people with median CD4 counts of 574 cells/µl blood	CD4 count (cells/µl blood), HIV Viral load (copies/ml blood)	• Increased HIV viral load in participants with higher AF-alb levels • Compared to participants in quartile 1, viral load was 2.3X more likely in quartile 3	Jolly *et al.*, 2013

Table 16 (cont'd)

AF-alb range (pg/mg albumin): Quartile 1: 0.20–4.97 Quartile 2: 4.98–10.63 Quartile 3: 10.64–20.27 Quartile 4: 20.28–109.87		participants and 2.9X more likely in quartile 4 participants • Lower mean CD4 cell count observed in Quartile 4 participants compared to participants in the other three quartiles (not statistically significant)	
A cohort of 472 Gambian children Age: 6-9 years (AF-alb range: 5–456 pg/mg with mean level of 22.3 pg/mg) Method: ELISA	Secretory IgA (sIgA) in saliva, cell-mediated immunity (CMI), antibody responses to rabies and pneumococcal polysaccharide vaccine	• sIgA was significantly lower in children with detectable AF-alb [50.4 µg/mg protein (95% CI: 48.0–52.8) compared with those with nondetectable levels [70.2 µg/mg protein (95% CI: 61.1–79.2); p< 0.0001 • Antibody response to one of four pneumococcal serotypes, but not rabies vaccine, was weakly associated with higher levels of AF-alb (P=0.05) • There was no association between cell-mediated immunity responses and AF-alb.	Turner *et al.,* 2003

Table 16 (cont'd)

A cohort of 391 Gambian children Age: 3-8 years (AF-alb range: 5-719.6 pg/mg of albumin Method: ELISA and HPLC	Antibodies to asexual malaria parasites and Hepatitis B surface antigen (HBsAg)	• Mean AF-alb adduct level was significantly higher in children with P. falciparum parasitaemia compared to children with no parasitaemia (P=0.011) • Mean AF-alb adduct levels were significantly higher in HBsAg positive children compared to the controls (P=0.04)	Allen *et al.*,1992
A cross-sectional study including 409 Kenyan children between the ages of 1–14 years AF-alb range: 0.74–901.15 pg/mg of albumin	Hepatitis B surface antibodies, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN- γ	• 98 out of 205 children (47.8%) tested positive for Hepatitis B surface antibodies • Anti-HBs dropped by 0.91 mIU/ml per unit rise in serum aflatoxin level • IL2, IL-6, IL-8 and IFN- γ cytokines showed a negative correlation with respect to aflatoxin blood levels (not statistically significant) • IL-10, TNF-α and GM-CSF showed positive correlation with respect to aflatoxin blood levels (not statistically significant)	Githang'a *et al.*, 2019b

Table 16 (cont'd)

A case study including cord blood samples from 3 GD patients and 3 controls AFB_1 (pg/ml) levels: Control (44 ± 3) GD patients (5471 ± 1606)	IL-10 cytokine	• IL-10 levels in cord blood samples of AFB_1 exposed GD patients were significantly up-regulated (865.42 ± 21.85 pg/ml) compared to non-GD controls (403.91 ± 56.18 pg/ml) ($P < 0.05$);	Xie *et al.*, 2018

Table 17: Animal studies of the effects of aflatoxin exposure on immune system markers.

Animal	**Aflatoxin dose and duration of experiment**	**Immune system biomarkers analyzed**	**Results**	**Reference**
C57BL/6 mice Age: 10 weeks	Single oral dose of 44, 442 or 663 μg AFB_1/kg of BW on day 1. Analysis conducted on day 5	Cytokine expression levels (IL-4, IFN-γ, and IL-17), the proliferative response for Con-A-stimulated lymphocytes	• 663 μg AFB1/kg BW-induced upregulation of cytokine expression levels (IL-4 and IFN-γ). • No significant difference in IL17 levels • 442 and 663 μg AFB1/kg BW significantly suppressed the proliferative response for Con-A-stimulated lymphocytes	Ishikawa *et al.*,2017

Table 17 (cont'd)

Balb/c mice Age: not reported	Oral administrated with 100 µg AFG_1 /kg BW for 1, 3 and 6 months	CD68+ macrophages, mononuclear cells, CD3+ lymphocytes, TNF-α, IL-1β, IL-6, MCP-1/CCL-2, MIP-2/CXCL-2 and CXCL-1, NF-κB, p-STAT3 and COX 2 expressions	• Increased CD68+ macrophages and CD3+ lymphocytes • Up-regulation of NF-κB and p-STAT3, and cytokines production. • TNF-α, IL-1ß, IL-6, MCP-1/CCL-2, MIP-2/CXCL-2 andCXCL-1 expressions were increased at the 3 different time points following AFG1 gavage ($p<0.05$).	Liu *et al.*,2015
Female mice from an inbred conventional mouse colony (ICR) Age: 4 months	Oral treatment (drink) with 200 µg AFB_1 /kg of BW over 24 days	CD3+ cells, WBC counts (leukocytes, lymphocytes, neutrophils and monocytes)	• Significant decrease in the number of $CD3^+$T cells in the intestinal mucosa of AFB_1 treated mice (65.75±5.36) compared to control group (82.67±2.36), $P< 0.05$ • Significant decrease in lymphocyte, neutrophils and monocyte counts in AF treated mice at $P< 0.05$. • No significant difference in leukocyte counts	Tomková *et al.*,2002
Male CD-1 mice Age: 7 weeks	Received 0, 30, 145 or 700 µg AFB_1/kg BW orally (gavage in	WBC counts, CMI, primary antibody response (IgM class	• AFB_1 exposure decreased peripheral WBC counts after 2 weeks dose-dependently	Reddy *et al.*,1987

Table 17 (cont'd)

	corn oil) every other day for 2 weeks in a corn oil: ethanol vehicle	antibody producing cells) of splenic lymphocytes against sheep red blood cells, T-independent antibody producing cells, delayed type hypersensitivity response (DTH) to keyhole limpet hemocyanin (KLH)	• AFB_1 exposure had no effect on RNA synthesis in splenic lymphocytes at any dose • AFB_1 exposure significantly decreased the number of IgM class antibody-producing cells per spleen in the medium and high dose groups • Number of T-independent antibody-producing cells was not altered by AFB_1 exposure • Exposed mice demonstrated a suppressed delayed-type hypersensitivity response to KLH	
Male BALB/c mice Age: not reported	Received 0, 30, 145 or 700 µg AFB_1/kg BW orally (gavage in corn oil) every other day for 4 weeks in a corn oil: ethanol vehicle	WBC counts, NK cell-mediated cytotoxicity of YAC-1 in splenic cells	• AFB_1 exposure decreased peripheral WBC counts after 4 weeks dose-dependently (significantly in the higher doses) • NK cell-mediated cytolysis was suppressed in a dose-dependent manner	Reddy and Sharma, 1989

Table 17 (cont'd)

Male CD-1 mice Age: 5 weeks	Mice were treated with 0, 30, 145 or 700 µg AFB_1/kg BW orally every other day for 2 weeks	WBC counts, cytokine mRNA levels of IL-lα, IL-6 and TNF produced by macrophages, (IL-2, IFNγ, and IL-3) produced by splenic lymphocytes	• WBC counts were significantly elevated at the low (30 µg AFB_1/kg BW) dose • Significant increase in the mRNA levels produced by macrophage at the low (IL-lα) or medium dose (IL-6 and TNF) • The low dose of AFB_1 slightly decreased mRNA expression levels of splenic lymphocytic IL-2 (significantly, $P<0.05$), IFNγ, and IL-3 (not significant)	Dugyala and Sharma, 1996
Male CD-1 mice Age: 6-8 weeks	400 µg AFB_1 /kg BW every other day for 2 weeks	Peritoneal macrophages, macrophage products (Nitric oxide (NO), Hydrogen peroxide (H_2O_2, superoxide anion (O_2^-)), TNF-α phagocytosis	• H_2O_2, NO and O_2^- productions in AFB_1 exposed group were reduced • TNF- α production in AFB_1 exposed group was reduced • Phagocytosis in AFB_1 exposed group was decreased	Moon *et al.*,1999 b
White mice Age: 60 days	Control diet and diets containing 200, 400, 800 and 1600 µg aflatoxin /kg BW	Leukocyte formula (proportions of lymphocyte, neutrophil, eosinophil, basophil, monocyte)	• Significant increase in proportion of neutrophils ($P<0.001$) • Proportion of eosinophils decreased significantly ($P<0.001$) • No significant change in basophil	Tuzcu *et al.*,2010

Table 17 (cont'd)

		and ANAE-positivity in peripheral blood	levels (except, significant decrease in the mice receiving 200 µg aflatoxin /kg BW) and monocyte levels • Lymphocyte prportions decreased significantly (P<0.001) in a dose-dependent manner Significant (P<0.001) decrease in the proportions of ANAE-positive peripheral blood lymphocytes	
Male Balb/c inbred mice Age: 6-8 weeks	Animals were dosed with 25 or 50 μg AFM_1/kg BW for 5 days a week for 4 weeks	Masses of spleen, thymus and their organ/BM ratios, total WBC counts, proliferation of lymphocytes, delayed-type hypersensitivity (DTH) response, subtypes of cells $CD19^+$, $CD49^b$, $CD3^+$, $CD4^+$ and $CD8^+$, (IFN)-γ, *IL-4* and IL-10, concentrations of IgG and IgM, total serum hemolytic activity, phagocytic	• No significant effects on spleen and thymus from AFM_1 • No significant differences in numbers of total WBC, lymphocytes, monocytes or neutrophils • Serum anti-SRBC titer indicated a significant suppression in AFM1 treatment groups compared to the negative control group • DTH was observed in mice exposed to AFM_1 compared to the negative control. • AFM_1 exposure suppressed the proliferative responses of	Shirani *et al.*,2018

Table 17 (cont'd)

		capacities of monocytes and granulocytes	splenocytes exposed to PHA or LPS • No significant difference in IL-4; Significant decrease in IFN-γ , while increase in IL-10 • Significantly lower CD3+, CD4+, CD8+ and CD19+ observed in spleens of mice exposed to 25 or 50 μg AFM1 /kg • % of CD3+ and CD8+ T-ymphocytes were lower in spleens • No significant difference in phagocytic activities observed • AFM1 did not affect the concentrations of IgM but concentrations of IgG in the blood serum of exposed-mice were significantly lower ($P< 0.001$)	
Male Kunming mice Age: 6 weeks	Control and 750 µg AFB_1/kg BW/day by intragastric administration for 30 days	Splenic CD3+, CD4+ and CD8+ T lymphocytes, Serum IL-2, IFN-γ and TNF-α content, spleen apoptosis rate	• Significant reduction in the proportions of CD3+, CD4+ and CD8+ T-lymphocytes in spleen ($P < 0.01$) • Significant reduction in IL-2, IFN-γ and TNF-α contents in serum and spleen	Xu *et al.*,2019

Table 17 (cont'd)

			• AFB1 treatment significantly increased the apoptosis rates of splenocytes compared to the control group ($P < 0.01$)	
Female mice Age: 4 months	Oral administration of control, 200 µg AFB_1/kg BW, "E. *cuniculi* + no AFB_1" and "200 µg AFB_1/kg BW + E. *cuniculi"* for 27 days	Total number of leukocytes, absolute number of lymphocytes, neutrophils and monocytes, CD4+ and CD8+ T cells in peripheral blood,	In AFB_1-treated group at 27 days: • Decrease in number of lymphocytes, monocytes, CD4+, CD8+ T cells (not significant compared to control) In AFB_1 + E. *cuniculi* group at 27 days: • Significant increase in monocytes compared to "E. *cuniculi* + no AFB_1" group • Decrease in no. of leukocytes, lymphocytes, neutrophils, CD4+ and CD8+ T cells (not significant compared to "E. *cuniculi* + no AFB_1" group"	Levkutová *et al.*,2003
Male F344 rats Age: 5 weeks	Control, 5, 25 and 75 µg AFB_1/kg BW (gavage) 1 or 5 weeks, 5 days a week	Splenic lymphocyte surface markers (CD3, CD4, CD8 and CD45R), combination of cell-surface markers	<u>1 week</u> • Dose-dependent decreases in the % CD8+ and CD3-CD8a+ NK cells; significant decrease in 25 and 75 µg/kg BW groups ($P<0.05$)	Qian *et al.*,2014

Table 17 (cont'd)

		and cytokine markers (CD4APC + CD8a PERCP + IL-4 PE + IFNγFITC; and CD3 PE+ CD8aPERCP + TNF-α FITC), splenic lymphocyte phenotype or cytokine expression	• Dose-related and significant reduction of IL-4 expression by CD4+T cells at all dose levels • Dose-dependent inhibition of IFN-γ expression by CD4+T cells; significant decrease in 75 µg/kg BW group • Significant inhibition of IL-4 and and IFN-γ expression by CD8a+cells in the 25 and 75 µg/kg BW groups <u>5-weeks</u> • Significantly increase in % of CD3(+) and CD8(+) T cells in the 5 and 25 µg/kg groups. • Significant decrease in IL-4 expression by CD4(+) T cells and significantly increase in IFN-γ expression by CD4(+) (only in 25 µg/kg BW group) • Significant increase of TNF-α expression by CD3-CD8a+NK cells (85.9%) in the75 µg AFB_1/kg BW group	

Table 17 (cont'd)

Adult male Brown Norway (BN) rats Age: not reported	Oral administration of 100 µg AFB_1/kg BW and 1000 µg AFB1/kg BW once a week for five weeks with and without OVA	CD4/CD8 ratio, expression of CD25 and CD71 activation markers of mesenteric lymphocytes, anti-OVA IgE, and -IgG antibodies	• In both low and high dose groups, no significant difference in CD4/CD8 ratio • No significant difference in the expression of activation markers on mesenteric CD4+ and CD8+ lymphocytes • High AfB1 + OVA group, there was an increased number of CD8+ and CD8/CD71+ cells in mesenteric lymph nodes indicating activation of T suppressor cells • In the low dose group, no effect observed on the ratio of CD4/CD8+ lymphocytes and on the percentage of CD4+ and CD8+ lymphocytes in mesenteric lymph nodes • No change in the serum concentrations of OVA specific IgE and IgG antibodies in both low and high dose groups	Watzl *et al.*,1999
Adult male Sprague-Dawley rats	200 µg AFB_1/kg feed ≈ 30 µg AFB_1/kg BW/day for 8 weeks	Total WBC counts in blood, lymphocytes, neutrophils, monocytes, eosinophils	• Aflatoxin treated group showed: ○ Significant reduction in total WBC count	Essa *et al.*,2017

Table 17 (cont'd)

		and basophils counts, neutrophils phagocytic activity, macrophage phagocytic activity, serum lysozyme activity, globulin (α, β, γ) levels	○ compared to control (P<0.05) ○ Significant decrease in lymphocyte and monocyte counts (P<0.05) ○ Significant increase in neutrophil count (P<0.05) ○ No change in eosinophils and basophils ○ Significant decrease in both neutrophils and macrophage phagocytic activity (P<0.05) ○ Significant reduction in serum lysozyme activity • Significant decrease in albumin and globulins levels	
Fisher-344 male rats Age: 21–24 days	0, 10, 40, 400, or 1600 µg AFB_1/kg diet ≈ 0, 6.45, 26, 258 or 1032 µg AFB_1/kg BW/day (4 weeks on and 4 weeks off for 40 weeks)	WBC differential counts (lymphocytes, segmented leukocytes, eosinophils, basophils, and monocytes), CD3, CD4, and CD8 or CD45R (B cell), IL-1, IL-2, and IL-6	• Total WBC count increased (p< 0.05) in continuously treated group (after 8 weeks) and in the intermittently group (after 12 weeks). • Increase in lymphocytes % and decrease segmented neutrophils %	Hinton *et al.*,2003

Table 17 (cont'd)

	plus, an additional group feeding on 1600 µg AFB_1/kg diet (≈ 1032 µg/kg BW/day) continuously		in group continuously treated group (after 12 weeks) • The % of CD3+ lymphocytes increased (28 to 57%), % of CD45R+ cells decreased (58 to 29%), % of CD4+ cells increased (20 to 49%), % of CD8+ cells remained unchanged • T-cells % significantly increased at the higher doses for both continuous (C) and intermittent (I) groups • B cell % significantly decreased at the higher dose groups compared to control • IL-1 and IL-6 levels significantly increased in the second dosing cycle (12 weeks) and the second "off" cycle (16weeks) at higher doses	
Adult male Wistar rats Age: Not reported	Control and 300 µg AFB_1/kg BW orally for two weeks on alternate days; total seven doses	Cellularity of spleen, thymus and bone marrow cells, phagocytic ability of the peritoneal macrophages, delayed-	• Significant reduction in cell counts of thymus and bone marrow ($P<0.001$) • Severely depleted peritoneal exudate cell population (by 40%)	Raisuddin *et al.*,1994

Table 17 (cont'd)

		type hypersensitivity response, lymphocyte count (T- and B-cells)	• 50% reduction in phagocytic function • Suppression in delayed type of hypersensitivity (in terms of foot pad thickness) response. • Significant depression in mitogenesis of T- nd B-cells ($P<0.001$)	
Crossbred weanling pigs Age: 21-days old	Diet containing 140 or 280 µg aflatoxin/kg feed ≈ 40 or 80 µg AFB_1/kg BW/day For 3 weeks	Total serum IgM and IgG concentrations, antibody response to sheep red blood cells (SRBC)	• Total serum IgM and IgG levels were not affected by either dose • No difference was observed in antibody response to SRBC in AFB_1 -treated group	Van Heugten *et al.*,1994
Weanling piglets Age: 4-weeks old	Diet containing 140 or 280 µg aflatoxin/kg feed (70% AFB_1 in total aflatoxin) ≈ 30 or 60 µg AFB_1/kg BW/day	Number of lymphocytes, neutrophils, monocytes, basophils, and eosinophils, IL-2, IL-4, IL-1β, TNF-α and IL-10, γ-globulin concentration in the serum	• No effect on the number of lymphocytes, monocytes, neutrophils, basophils, and eosinophils in blood • No effect of IL-2 and IL-4 • Decreased IL-1β, TNF-α and increased IL-10 cytokine mRNA expression • Biphasic effect on total WBC count; 140 µg aflatoxin/kg feed dose decreased the total number of	Marin *et al.*,2002

Table 17 (cont'd)

			• WBC but 280 µg aflatoxin/kg feed increased total WBC count • Increased concentration of γ-globulin in the serum	
Pigs Age: 3-weeks old	Diets containing 385, 867 or 1807 µg AFB_1/kg feed for 28 days ≈ 73, 165 or 344 µg AFB_1/kg BW/day	Plasma concentrations of total IgA, IgG and IgM and anti-ovalbumin IgG, expression levels of TNF-α, IL-1β, IL-6, IFN-γ, IL-10 cytokines in spleen, WBC count (neutrophils and monocytes), antigen to ovalbumin	• A significant up-regulation of TNF-α, IL-1β, IL-6, IFN-γ and IL-10 cytokines was observed in spleen from pigs exposed to the highest dose of AFB_1 • No major change in plasma concentrations of total IgA, IgG and IgM and anti-ovalbumin IgG • Pigs exposed to the highest dose of AFB_1 also showed an increase in circulating neutrophils compared to the controls (11,371± 2697/ml versus 4790±462/ml); monocyte counts were not significantly different • Dose-dependent reduced lymphocyte proliferation was observed after stimulation with the vaccine antigen	Meisson nier *et al.*,2008

Table 17 (cont'd)

			• None of the three AFB1 contaminated diets affected the anti-OVA IgG production	

Figure 10: Selection of studies for inclusion in systematic review of aflatoxin-associated immunomodulation

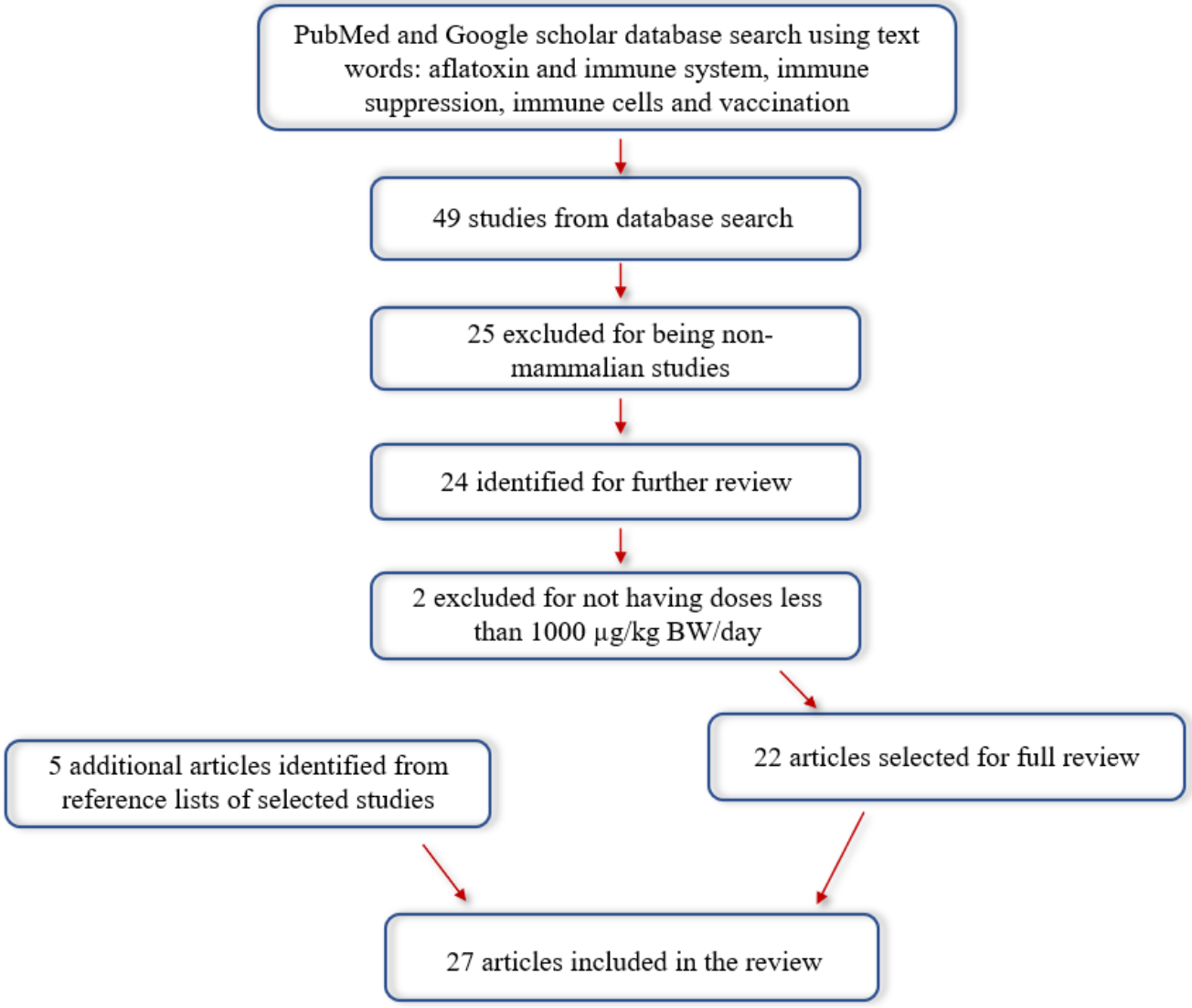
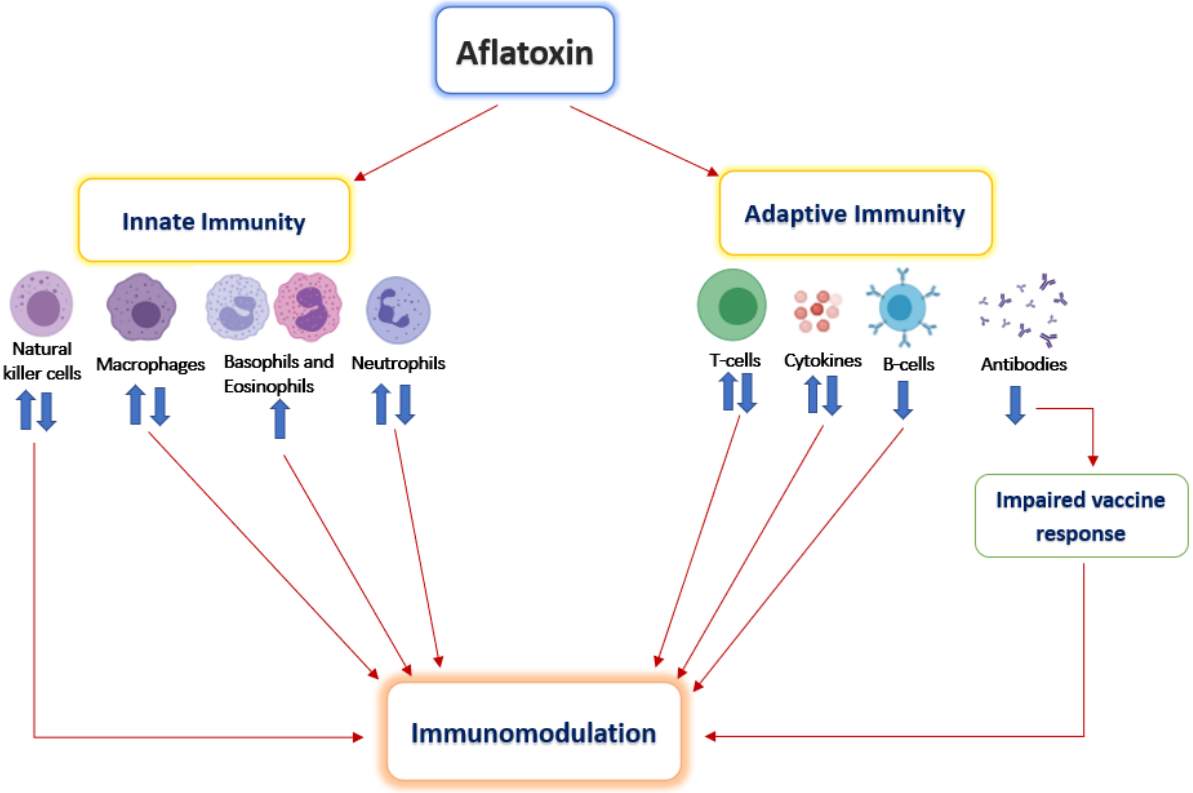


Figure 11: Effects of aflatoxin exposure on immune system components. (Created with Biorender.com)



Note: ⬆ = upregulation, ⬇ = downregulation

APPENDIX B: Risk assessment of aflatoxin-related liver cancer in Bangladesh

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Abstract

Aflatoxins are mycotoxins (fungal toxins) produced by Aspergillus species in variety of food commodities. Consumption of aflatoxin-contaminated food can cause adverse health effects, including liver cancer. Aflatoxin exposure is usually higher in hot and humid countries. Previous biomarker-based studies have indicated significant exposure to aflatoxins among the Bangladeshi population. Recently, high aflatoxin levels were reported in dates, which are consumed in large quantities during the month of Ramadan in Bangladesh and other Muslim countries. Bangladesh has recently enacted aflatoxin regulation in foods. In this study, we determined the risk of aflatoxin-related liver cancer among the Bangladeshi population based on the average dietary intakes of different aflatoxin contaminated foods, accounting for the synergistic impacts of aflatoxin with chronic hepatitis B viral infection in inducing cancer. We also determined whether the new aflatoxin regulations in Bangladesh could significantly reduce the risk of liver cancer. The mean number of cancer cases per year caused by dietary aflatoxin exposure in Bangladesh was estimated at about 1311, or 43.9% of the total annual liver cancer cases in Bangladesh. The new aflatoxin regulations do not appear likely to significantly reduce the risk of liver cancer in the country.

Key Words: Aflatoxin exposure, risk assessment, liver cancer, Bangladesh, food safety regulations

Introduction

Aflatoxins are toxic secondary metabolites of the fungal species Aspergillus flavus and A. parasiticus, which colonise a wide variety of food crops such as maize, groundnuts, tree nuts, various spices, and cottonseed (Alshannaq *et al.*,2017). Factors that influence aflatoxin production are drought stress, rainfall, insect damage, crop genotype, and agricultural practices in the field and in storage (Khlangwiset and Wu 2010). The four major derivatives of aflatoxins are AFB1, AFB2, AFG1, and AFG2. AFB1 is the most common one in food, and the most toxic and carcinogenic. Chronic exposure to AFB1 increases the risk of liver cancer, or hepatocellular carcinoma (HCC) in humans and multiple other animal species (Kew 2013). In the body, AFB1 is metabolized into a reactive exo-8,9-epoxide form in the liver by Cytochrome P450 enzymes. The exo-epoxide reacts with DNA to form an AFB1-DNA adduct, causing DNA mutation and increased liver cancer risk (Kensler *et al.*,2011; Kew *et al.*,2013). These compounds have been evaluated on several occasions by the International Agency for Research on Cancer (IARC), including many experimental and human studies that have confirmed their carcinogenic properties. IARC has classified 'naturally occurring mixes of aflatoxins' as Group 1 human carcinogen (IARC 1993). People who are chronically infected with hepatitis B virus (HBV) have a 30-fold higher risk of developing hepatocellular cancer from aflatoxin consumption than those who are HBV-negative (JECFA 1998). High doses of aflatoxin can also result in acute aflatoxicosis, characterized by hemorrhage, acute liver damage, and even death. Aflatoxin exposure has also been linked to immune dysfunction and growth impairment in children and multiple animal species (Khlangwiset *et al.*,2011; Wu *et al.*,2014; Mitchell *et al.*,2017). Moreover, food production can be negatively impacted by high aflatoxin levels, resulting in natural resource waste, significant economic losses, and limitation in the development of international trade due to the existing strict

regulations in high value markets (Udomkun *et al.*,2017). Today, food-borne aflatoxin is regulated in over 100 nations worldwide. Several countries regulate total aflatoxins (B1+ B2+ G1+ G2), several regulate only AFB1, and several regulate both total aflatoxins and AFB1 in their food commodities. The number of countries establishing limits on aflatoxin levels has been increasing since 1995 (Pinstrup-Andersen and Cheng 2009). In the United States, the action level for maximum allowable aflatoxin in human food is 20 μg aflatoxin per kg food (USFDA 2000). The purpose of this paper is to assess the impact of the new, recent aflatoxin regulations set by the Bangladesh government, on liver cancer risk in its population. High temperature with high humidity and cycles of drought followed by heavy rainfall are conductive to aflatoxin accumulation in crops (Wagacha and Muthomi 2008). Bangladesh is a tropical country and has frequent occurrence of cyclical drought and flooding: conditions conducive to growth of Aspergillus and accumulation of aflatoxins (Roy *et al.*,2013). A recent study that analyzed the AFB1-lysine adduct in women's serum from the first and third trimester of pregnancy, and in their children at 24 months of age, indicated a high risk of exposure for the population in Bangladesh (Groopman *et al.*,2014). Another study that investigated the occurrence of urinary AFM1 (a biomarker of short-term aflatoxin exposure) in two adult cohorts (rural and urban) in Bangladesh found significant aflatoxin exposure in both populations (Ali *et al.*,2016).

In Bangladesh, there were no regulations for aflatoxins in food until July 2017, when the Bangladesh Food Safety Authority set regulations for total aflatoxin contamination in different kinds of nuts (groundnuts, almonds, Brazil nuts, hazelnuts, and pistachios) up to maximum levels of 10 μg/kg for direct consumption (BFSA 2017). This study was conducted to determine the risk of aflatoxin-related liver cancer based on consumption of aflatoxin-contaminated food among the Bangladeshi population, and to evaluate whether the current aflatoxin regulations in Bangladesh

result in a significant decrease in liver cancer risk, or if more strict regulations are necessary, for example, limiting aflatoxin levels in not only nuts but also in other food commodities prone to aflatoxin contamination.

Materials and methods

Data collection

We employ the quantitative cancer risk assessment methodology laid out in our previous study (Liu and Wu 2010). For this risk assessment for the Bangladeshi population, we used aflatoxin occurrence data in multiple human foodstuffs from Roy *et al., (*2013) and Bhuiyan *et al., (*2013) (outlined in Table 18). Roy *et al., (*2012) analyzed AFB1 levels found in dates, groundnuts, lentils, spices, rice, and wheat in Bangladesh; we multiplied the AFB1 values by two to derive estimates for total aflatoxin (AFB1 + AFB2 + AFG1 + AFG2) levels in these food commodities. Bhuiyan *et al., (*2013) found total aflatoxin levels in maize, wheat, and rice collected from different market stalls in Bangladesh. For wheat and rice, we have considered the aflatoxin levels determined by both Roy *et al., (*2012) and Bhuiyan *et al., (*2013) by calculating the geometric mean, minimum, and maximum values, using the total aflatoxin levels determined by Bhuiyan *et al., (*2013) and our estimated total aflatoxin levels (multiplying AFB1 levels by 2, following the rule of thumb that total aflatoxin levels are about twice the level of AFB1) determined by Roy *et al., (*2012).

Roy *et al., (*2012) used HPLC (Micro-tech Ultra-Plus II Micro LC System) to analyze AFB1 levels with 0.2 μg/kg LOD and 89% recovery, using 5 to 10 pooled samples for each commodity collected from three different sites in Bangladesh (Dhaka, Chittagong and Sirajgonj) in September 2009. Bhuiyan *et al., (*2013) analyzed the total aflatoxin levels in maize, rice, and wheat using HPLC (Agilent series 1100) with 0.5 μg/kg limit of detection (LOD) and 87–92% recovery, using 180 samples of each commodity collected from all six districts of Bangladesh (Dhaka, Rajshahi,

Chittagong, Sylhet, Khulna, Barisal), and at six different times of the year. For our risk assessment purposes, we have considered only the minimum and the maximum aflatoxin concentrations detected in maize, wheat, and rice.

Table 18: Total aflatoxin levels in different food commodities in Bangladesh.

Commodity	**Total aflatoxin contamination C (µg/kg)**			**Data source**
	$C_{Minimum}$	**C_{Mean}**	**$C_{Maximum}$**	
Dates	5	224	1246	Roy *et al., (*2012). AFB_1 levels were doubled to estimate total aflatoxin levels.
Groundnut	3.6	186.2	846	
Lentils	9.6	42.4	85	
Red chilli	>40	>40	>40	
Wheat [a]	1.34	6.65	28.28	Geometric mean of total aflatoxin levels from Bhuiyan *et al., (*2013) and Roy *et al., (*2012).
Rice [a, b]	0.45	2.58	14.940	
Maize [a]	3	27.66	255	Bhuiyan *et al., (*2013).

[a]The C_{mean} of total aflatoxin levels for rice, wheat and maize were calculated using the Geometric mean of the minimum and maximum levels.

[b]For rice, the minimum AFB_1 level detected by Roy *et al., (*2012) was below the limit of detection (LOD) and was assumed to be half of the LOD for further calculations.

Food consumption data

The average dietary consumption data for each of the food commodities were obtained from FAOSTAT (2013). This database estimated the average adult consumption of each foodstuff in Bangladesh in grams/day during a three-year period.

Exposure assessment

The minimum, mean, and maximum lifetime average daily doses (LADD) were calculated

based on the minimum, mean, and maximum aflatoxin concentrations in the food commodities by using the formula:

$$LADD_{(min/mean/max)}\ (\mu g/kg\ BW/day) = (IR_{average} * C_{(min/mean/max)})/\ BW$$

where LADD = Lifetime Average Daily Dose, $IR_{average}$ = daily average Intake Rate (kg/day), C = Concentration of aflatoxin in food (µg/kg), and BW = Body Weight of the global-average adult, 70 kg. The C_{mean} values for rice, wheat, and maize were calculated using the geometric mean of the $C_{minimum}$ and $C_{maximum}$ values.

Table 19: Dietary exposure assessment of aflatoxin in Bangladesh.

Commodity	**Amount consumed per day (g)**	**IR_{avg} (kg/day)**	**C Min (µg/kg)**	**C Mean (µg/kg)**	**C Max (µg/kg)**	**$LADD_{min}$ (µg/kg bw/day)**	**$LADD_{mean}$ (µg/kg bw/day)**	**$LADD_{max}$ (µg/kg bw/day)**
Dates	0.1	0.0001	5	224	1246	0.00001	0.00032	0.00178
Groundnut	0.22	0.0002	3.6	186.2	846	0.00001	0.00059	0.00266
Lentils	10.12	0.0101	9.6	42.4	85	0.00139	0.00613	0.01229
Chilli/spices	5.36	0.0054	>40	>40	>40	0.00153	0.00153	0.00153
Wheat	47.86	0.0479	1.34	6.57	28.28	0.00092	0.00455	0.01934
Maize	2.18	0.0022	3	27.66	255	0.00009	0.00086	0.00794
Rice	470.49	0.4705	0.45	2.59	14.94	0.00301	0.01737	0.10042
Total dietary exposure of aflatoxin per day						**0.00696**	**0.03135**	**0.14596**

IR = intake rate, C = concentration of aflatoxin in the food commodity, LADD = lifetime average daily dose of aflatoxin from each of the food commodities. Min = minimum, Max = Maximum

Risk characterization

This final step of risk assessment integrates dose-response and exposure data to describe the overall nature and magnitude of risk. For our study, this final step consisted of quantifying the burden of aflatoxin-related liver cancer for the whole population of Bangladesh, accounting for those both with and without chronic HBV infection. As per our earlier study Liu and Wu (2010), we estimated total number of individuals with or without chronic HBV by multiplying prevalence (5.4% in Bangladesh (Mahtab 2015)) by population size (163 million (The Commonwealth 2016)). To estimate aflatoxin-induced HCC rates within these two populations (with and without chronic HBV infection), we multiplied the corresponding JECFA cancer potency factor by aflatoxin exposure estimates. We summed across both HBV+ and HBV– individuals to arrive at an estimate for total burden of aflatoxin-induced HCC in Bangladesh.

Results

Table 19 shows the minimum, mean and maximum aflatoxin exposure levels from each of the food commodities which are calculated using the formula for LADD described above. The highest mean aflatoxin exposure level (17.37 ng/kg BW/ day) was from rice due to its high intake (up to 60% of the energy intake in Bangladeshi diet). However, the aflatoxin exposure levels from other foods were relatively lower. In Bangladesh, the mean aflatoxin concentrations found in rice and in wheat, which are the two main staple foods in Bangladesh, fell below the US aflatoxin regulatory limit of 20 μg/kg. However, in some commodities such as maize, groundnut, lentils, dates, and red chili, the mean aflatoxin levels (27.66 μg/kg, 186.2 μg/kg, 42.4 μg/kg, 224 μg/kg, and >40 μg/kg respectively) exceeded the US regulatory limit. The highest average concentration of aflatoxin among these food commodities was found in dates (224 μg/kg), but its average daily intake was

only 0.1 g/day. However, dates are consumed in high amounts during the Islamic month of fasting so the average daily intake would be much higher during the month of Ramadhan. The average aflatoxin concentration found in groundnuts was also much higher (186.2 μg/kg) than the regulatory standards; however, it must be currently under control since aflatoxin levels in nuts are now regulated in Bangladesh. Based on the food consumption data and the available aflatoxin level data in these food commodities (Table 19) the average daily intake of aflatoxin in Bangladeshi general population through food consumption was estimated to be 31.35 ng/kg BW/day. This estimation is relatively high compared to the average daily intake of aflatoxin in Europe (0.93–2.4 ng/kg bw/day) and United States (2.7 ng/kg bw/day) but falls in the range of that in Asia (0.3–53 ng/kg bw/day) and in Africa (3.5–180 ng/kg bw/day) (JECFA 2007).

Table 20 shows the overall risks of liver cancer cases in Bangladesh due to aflatoxin exposure for both HBV (−) and HBV (+) individuals. The minimum number of estimated cancer cases in Bangladesh among HBV (−) individuals was 107 cancers/year and the mean number was 483 cancers/year. In terms of HBV (+) individuals, the minimum estimated cancer cases were 184 cancers/year and the mean number was up to 828 cancers/year. With the current aflatoxin regulation in nuts, the mean estimated number of liver cancer cases per year only goes down by 1.8% (Table 20). Therefore, the current aflatoxin regulation in nuts is not reducing the number of cancer cases per year significantly in the country since the average daily intake of nuts and nut products is very low.

According to GLOBOCAN 2012 (the International Agency for Research on Cancer database for global and national cancer estimates), the number of liver cancer cases in Bangladesh per year is 3022. Based on our calculations, aflatoxin exposure alone (after current regulatory limits) may contribute to 43.9% of the total estimated liver cancer cases in Bangladesh, taking into account the

synergism of aflatoxin and HBV in causing liver cancer. This is significantly higher than the global average of aflatoxin-related liver cancer cases in our previous estimates (Liu and Wu 2010; Liu *et al.*,2012), in which aflatoxin with or without HBV accounted for 5–28% of liver cancer cases. The difference could be accounted for by either underreporting of liver cancer cases registered in GLOBOCAN, or truly a higher risk among the Bangladeshi population from aflatoxin-related cancer due to the relatively high HBV rate and the aflatoxin contamination in commonly consumed foods.

Table 20: Estimated additional number of liver cancer cases in Bangladesh per year

	HBV (-) min	**HBV (+) min**	**HBV (-) mean**	**HBV (+) mean**	**HBV (-) max**	**HBV (+) max**
Estimated additional Number of Liver Cancer Cases/year in Bangladesh	107	184	483	828	2251	3853
Number of liver cancer cases/year for HBV- and HBV+ combined	291		1311		6104	

Table 21: Annual HCC cases before and after current aflatoxin regulation in Bangladesh.

	Annual liver cancer cases			
	HBV (-) mean	**HBV (-) maximum**	**HBV (+) mean**	**HBV (+) maximum**
Before regulation	483	2251	828	3853
After regulation	475	2210	813	3784

Discussion

The mean total aflatoxin levels in most of the food commodities such as maize, lentil, dates, red chili spice, and groundnuts were higher than the US regulatory levels of 20 μg/kg. However, the two main staple food in Bangladesh, wheat and rice, had comparatively lower levels of aflatoxin and were within the range of maximum US regulatory levels. Nevertheless, the occurrence of high

levels of aflatoxin especially in lentils and red chili spices might be a matter of concern since these are consumed on a daily basis in Bangladesh and it would be ideal for the food regulatory system to consider enforcing aflatoxin regulation in the food items that are consumed regularly in Bangladeshi diet. The highest mean aflatoxin contamination (224 μg/kg) was found in dates, which are consumed regularly during the Islamic month of Ramadhan among the Muslim population. Therefore, Bangladesh may also consider monitoring aflatoxin in dates, and setting regulatory limits for this food. The current aflatoxin regulation in groundnuts of up to 10 μg/kg does not make a significant difference in number of annual liver cancer cases, since the average daily intake of nuts and nut products is very low among the Bangladeshi population. Moreover, the regulation would not necessarily cover nut products sold by street food vendors, which are popular throughout Bangladesh. According to our study, aflatoxin exposure alone may cause between about 291 to 6100 liver cancers per year with an average of 1311 cancers per year, considering both HBV (+) and HBV (-) combined individuals in Bangladesh based on the average dietary intakes of different food commodities contaminated with aflatoxins. This accounts for 43.9% of the total estimated liver cancer cases in Bangladesh which is 3022 per year (GLOBOCAN 2012). In our previous studies we estimated the global average of aflatoxin-related liver cancer cases to be 5–28% (Liu and Wu 2010; Liu *et al.*,2012). However, based on the results of our current study, the percentage of average aflatoxin-related liver cancer cases is significantly higher in Bangladesh compared to the global average.

Even though the average daily intakes are low for the food commodities with higher aflatoxin concentrations, the Bangladeshi population is still at a significant risk from aflatoxin exposure. The incidence of the liver cancer caused by aflatoxin can be reduced by decreasing human exposure level to aflatoxin that can be achieved by augmenting the regulation of aflatoxins in food

commodities of interest, as had been previously shown in a human risk assessment of aflatoxin in Korea (Lee *et al.*,2009). Also, there are several technologies that are already developed to control aflatoxin contamination in crops including biological control and chemical control, irradiation, ozone fumigation, and improved packaging materials (Udomkun *et al.*,2017). Bangladesh may consider adopting these aflatoxin control interventions which not only can improve food security but also strengthen the country's economic sustainability.

One potential limitation of this risk assessment concerns whether the food samples gathered were representative of individuals' intake. Although we calculated aflatoxin-related liver cancer risk based on aflatoxin levels in market products, Bhuiyan *et al., (*2013) found some aflatoxin levels in farmer's stored food that exceeded these levels. For example, the maximum levels of aflatoxin in farmer's stored food were 241 μg/kg in rice and 280 μg/ kg in wheat. Therefore, it is possible that a higher level of aflatoxin-related HCC risk exists than is calculated here, because in rural areas of Bangladesh, much food is produced and stored at the household level without entering the market for potential regulation. Many Bangladeshi farmers, people living in the rural areas and people living in poverty may not be well aware of aflatoxin contamination and the risks associated with it being more prone to aflatoxin consumption. According to our present study, aflatoxin does appear to be one of the strongest contributors to liver cancers in Bangladesh.

Moreover, the recent biomarker-based assessment on aflatoxin exposure in Bangladeshi population by Groopman *et al., (*2014) and Ali *et al., (*2016) indicate significant aflatoxin exposure, which raises concerns about whether stricter surveillance of aflatoxin contamination in foods other than nuts (such as grains and dates) is advisable. Also, a study conducted in China found that, reducing dietary exposures to aflatoxin is likely to significantly reduce liver cancer risk, even in those who are already infected with HBV (Chen *et al.*,2013). HBV affects almost 9

million people in Bangladesh and is the leading cause of liver cancer in this country, causing 47–61% of cases (Al-Mahtab 2015). Since liver cancer risk becomes multiplicatively higher for individuals exposed to both aflatoxin and chronic HBV, it may be a reasonable decision to have more strict regulation of aflatoxins in Bangladesh; incorporating more commodities regulated and monitored for this toxin based on the foods most commonly consumed by the Bangladeshi population.

APPENDIX C: Aflatoxin M1 in milk: A global occurrence, intake, & exposure assessment

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Abstract

Background: Aflatoxin B1 (AFB1), a naturally occurring mycotoxin (fungal toxin) in maize and nuts, causes liver cancer and has been associated with other adverse health effects. Much less is known about the toxicity of its metabolite AFM1, which is secreted in the milk of mammals. Nonetheless, many nations have set regulatory limits for maximum allowable AFM1 in milk and other dairy products.

Scope and approach: We collected comprehensive data on the occurrence of AFM1 in samples of milk worldwide, encompassing a wide range of different milk types: raw, pasteurized, ultra-high-temperature treated, fresh, and powdered. For each nation, we found average daily milk intake based on national or global dietary surveys. We then used the AFM1 concentration data and intake rates to calculate AFM1 exposure for adults in multiple nations worldwide.

Key findings and conclusions: Several nations including Pakistan, India, and several sub-Saharan African nations, had AFM1 levels in milk that substantially exceeded United States and European Union regulatory limits for AFM1, indicating potential risk to individuals in those nations with high milk consumption. Because no regulatory agency has set a tolerable daily intake (TDI) for AFM1, we could not compare our exposure estimates to a TDI to determine at-risk populations. But importantly, high AFM1 levels in milk indicate high levels of AFB1 in animal feed. This may imply that the crops used to make that feed such as maize, which humans might also consume, may have high AFB1 levels that could harm human health.

Keywords: aflatoxin M1, milk, dairy products, occurrence, intake rate, exposure assessment

1. Introduction

The objective of this work is to estimate human exposures worldwide to aflatoxin M1 (AFM1) through milk consumption. AFM1, a hydroxylated metabolite of aflatoxin B1 (AFB1) in human food and animal feed, is excreted in urine and secreted in milk in mammalian species. Aflatoxin B1, as well as aflatoxin B2, G1, and G2, are mycotoxins (fungal toxins) produced by *Aspergillus flavus* and *A. parasiticus* when they colonize food and feed crops such as maize, peanuts, cottonseed, sunflower seeds, and tree nuts (Wu *et al.*,2014, Alshannaq and Yu 2017, Mmongoyo *et al.*,2017). For nearly sixty years, AFB1 has been known to cause liver cancer in humans and other animal species. The International Agency on Research on Cancer (IARC) has classified AFB1 as group 1 human carcinogen (IARC, 2002). Now, AFB1 is also being associated with other adverse health effects such as child growth impairment, immune dysfunction, and acute toxicosis at high doses (Azziz-Baumgartner *et al*.2005; Bondy & Pestka 2000; Khlangwiset *et al.*,2011; Smith *et al.*,2017; Strosnider *et al.*,2006; Wild *et al.*,2015).

Exposure to AFM1 mainly occurs through consumption of contaminated milk (IARC, 1993). AFM1-induced acute hepatotoxicity was initially observed in a duckling study where the birds were orally exposed to AFM1 (Purchase 1967). AFM1 alone can also cause damage to DNA by covalently binding to it (Shibahara *et al.*,1995), which may enhance the genotoxicity already caused by AFB1 (Ben Salah-Abbes *et al.*,2015). AFM1 has also demonstrated a direct toxic potential in human cell lines, that too in absence of a metabolic activation (Neal *et al.*,1998). Several *in vivo* studies have also indicated suppressive effects of AFM1 on both innate and

adaptive immune responses (Shirani *et al.*,2018; Shirani *et al.*,2019). AFM1 was classified as a possible human carcinogen (group 2B) by IARC (IARC, 1993).

Since milk and milk products are daily consumed in many parts of the world and they are especially important in the diets of children, who may be more vulnerable to adverse effects from AFM1 (Galvano *et al.*,1996), multiple nations around the world have enacted food safety regulations for the presence of AFM1 in milk and other dairy products. The regulations are primarily to protect any market from contaminated food products to ensure health of consumers, based in part on assuming: (1) AFM1 has a toxicity similar to that of AFB1, and (2) a presence of AFM1 in dairy products is proportional to AFB1 exposures in dairy animals through their feed. The United States Food and Drug Administration (FDA) has set an AFM1 action level in milk and other dairy products at 0.5 µg/L; based in part on the FDA action level for total allowable aflatoxins (AFB1 + AFB2 + AFG1 + AFG2) in food and feed: 20 µg/kg; implying that the amount excreted as AFM1 as a proportion of total aflatoxins in feed is 2.5%. This is within the range estimated by van Egmond and Dragacci (1-6% AFB1: AFB1 makes up about half of total aflatoxins; 2001). The European Union (EU) has a much stricter AFM1 standard: it allows a maximum of 0.05 µg/L AFM1 in milk, however, no clear limit is set for other dairy products. This has led to incidents over the last decade of milk being dumped or production halted in various European nations because of AFM1 levels that exceeded 0.05 µg/L (DutchNews 2013, Whittle 2013). Whether there is a significant health benefit of enacting such a strict standard is, in the current state of knowledge on the toxicology of AFM1, unclear.

The economic consequences of AFM1 in milk and dairy products can be severe to dairy producers. A direct economic impact occurs when products that do not meet the aflatoxin standards are rejected at national or international markets (Balina *et al.*,2018). For example, Serbia had an

AFM1 outbreak in 2013 that resulted in product recalls and a dramatic reduction in purchases of milk and dairy products (Popovic *et al.*,2016). During this crisis in Serbia, which lasted almost two years, a total loss of up to 96.2 million EUR by the Serbian farm-level dairy sector was identified by Popovic *et al., (*2016). Senerwa *et al., (*2016) reported a possible economic cost for dairy feed manufacturers of $22.2 billion annually, and a further $37.4 million suffered by farmers due to reduced milk yield from cows fed aflatoxin-contaminated feed. These high financial losses have resulted in Serbian regulation for AFM1 to change from 0.05 µg/L to 0.5 µg/L (Serbian Regulation 2013). About 10% of the milk samples collected in Kenya contained aflatoxin levels above 0.5 µg/L, which would cost dairy farmers $113.4 million annually if legislation was enacted (Kemboi *et al.*,2020; Senerwa *et al.*,2016).

In this review, we estimate human exposure to AFM1 from liquid milk consumption in those nations. We report our findings from an extensive literature search on AFM1 levels measured from the year 2002 to 2020, in different types of liquid milk (raw milk, pasteurized milk, UHT milk etc.) and powdered milk (reported from 1994 to 2020), as well as average daily consumption of milk on a country-by-country basis. Considering, raw milk is consumed in many parts of South Asia and Africa and also because AFM1 is relatively resistant to any heat treatments (Galvano *et al*.,1996; Yousef and Marth, 1989), we have also included raw milk for our assessment. From the AFM1 concentration values in different types of milk (as well as taking into account non-detect samples of milk for AFM1), we conducted exposure assessment calculations for each country, and arrived at the average daily dose (ADD) of AFM1 for the average adult in each country.

2. Methods

Exposure assessment, one of the key stages of risk assessment, is the process of determining the amount of a chemical (or microbe, or other harmful agent) to which an individual or a population of interest is exposed; oftentimes measured in terms of milligrams or micrograms per kilogram bodyweight per day for dietary chemical exposures. For AFM1, our exposure assessment determined how much liquid milk adult populations across the world consume (AFM1 is typically not found in other food sources), and how much AFM1 is in those dairy products; hence, extrapolating to an average daily dose. Exposure is calculated as:

$$ADD = C_{ave} * IR / bw,$$

where *ADD* is the average daily dose (average daily exposure), C_{ave} is the average concentration of the toxin in the foodstuff of interest (e.g., µg AFM1 per kg of milk), *IR* is the intake rate by the individual of liquid milk, and *bw* is the individual's bodyweight. For a population, the average *IR* and *bw* are estimated.

PubMed and Google Scholar and PubMed search engine databases were searched using the key words: [aflatoxin M1], [AFM1], [milk], [occurrence], and specific country names to find studies that reported AFM1 levels in different types of milk in different countries around the world. When available, the percentage of samples containing detectable levels of AFM1, the range of AFM1 in the samples, the means and the limit of detection (LOD) were recorded from each study. If a study did not report a mean AFM1 level but provided the range of AFM1 levels detected in the samples, we calculated the means using the minimum and maximum values. For each study, we took into account the samples that had non-detectable levels of AFM1 by assuming the

minimum value to be one-quarter of the LODs, and calculated the mean using the following equation:

Mean for AFM1-positive and AFM1-negative samples combined = [(Mean)*(% of positive samples)] + [(LOD/4)*(% of negative samples)]

When multiple studies reporting AFM1 levels in milk were found from one country, we calculated the geometric means of the mean levels of AFM1 reported by all the studies from that particular country to determine a C_{ave} for AFM1 for that country.

Next, we obtained daily average consumption or IR of liquid milk by an adult in each country from the Food and Agriculture Organization of the United Nations (FAOSTAT) Food supply quantity database (FAOSTAT, 2017), the FAO/WHO Chronic individual food consumption database (CIFOCO) (WHO, 2017), WHO GEMS (Global Environment Monitoring System) Food Consumption database (WHO, 2012) and also individual studies reporting daily individual milk consumption when available. Finally, we calculated the *ADD* for each country using the calculated C_{ave} and *IR* values, assuming an adult bodyweight of 70 kg.

3. Results

Table 21 shows the data that we used for our exposure assessment of AFM1 in different countries of the world, from consumption of liquid milk. For this table, the milk types included: raw milk, pasteurized milk, "fresh milk," ultra-high-temperature (UHT)-treated milk, conventional milk, and organic milk. On a country-by-country basis, we describe the type of milk, the percentage of samples that tested positive for AFM1, the range of AFM1 in those positive samples, the reference for the study in question, daily consumption of milk in each of the nation, and finally – our

exposure calculations, measured in ADD – average daily dose, in µg/kg bw/day; assuming an adult bodyweight of 70 kg.

Table 22: Aflatoxin M_1 occurrence in different types of milk, and human exposures in different countries.

Country	Type of milk	% of AFM_1 positive sample	Range and mean of AFM_1 (µg/L)	Reference	Geomean for all milk types and studies (µg/L)	Daily consumption of milk (kg/ day)	ADD (ng/kg bw/day)
Algeria	Raw milk	46.43	0.096-0.557 Mean: 0.072	Mohammedi-Ameur *et al*., 2020		0.333 (FAOSTAT, 2017)	0.3425
Argentina	Raw milk	64	n.d-0.07 Mean: 0.028	Alonso *et al*., 2010	0.0095	0.081 (MinAgri, 2010) 0.435 (FAOSTAT, 2017)	0.0110 - 0.0591
	Pasteurized milk	50	Mean*:0.0078	Lopez *et al*., 2003			
	Farm milk	10.8	Mean*:0.0040				
Bosnia	Raw milk		0.001-0.06 Mean: 0.006	Bilandžić *et al*., 2016	0.0060	0.54 (FAOSTAT, 2017)	0.0463
	UHT milk		0.002-0.012 Mean: 0.006				

Table 22 (cont'd)

Brazil	Pasteurized milk	100	0.01-0.03 Mean: 0.02	Sifuentes dos Santosal *et al*., 2015	0.1231[a]	0.132 (IBGE, 2010) 0.394 (FAOSTAT, 2017)	0.2321 - 0.6934
		95.2	0.01-0.2 Mean: 0.031	Shundo *et al*., 2009			
		58.3	n.d-1.5 Mean: 0.884	Scaglioni *et al*., 2014			
	Raw milk	28.6	n.d- 1.7 Mean: 0.835				
	UHT milk	66.7	n.d-1.5 Mean: 1.168				
		87.5	n.d-0.121 Mean: 0.02	Silva *et al*., 2015			
		100	0.01-0.08 Mean: 0.04	Sifuentes dos Santosal *et al*., 2015			
China	Raw milk	4.64	n.d – 0.06 Mean: 0.015	Li *et al*., 2018	0.0252	0.066 (FAOSTAT, 2017)	0.0238
		75.2	0.0053–0.0362 Mean: 0.016	Xiong *et al*., 2020			
			Mean: 0.08	Huang *et al*., 2014			
	UHT milk	78.6	0.005–0.100 Mean: 0.015	Xiong *et al*., 2020			
		54.9	0.006-0.16 Mean: 0.0121	Zheng *et al*., 2013			
	Pasteurized milk	96.2	0.023-0.154 Mean*:0.0693				
		82.2	0.005–0.104 Mean: 0.027	Xiong *et al*., 2020			

Table 22 (cont'd)

Colombia	Pasteurized milk	79.3	0.011-0.289 Mean: 0.035	Diaz and Espitia, 2006		0.333 (FAOSTAT, 2017) 0.40 (Marimón Sibaja *et al*., 2019)	0.1665 -0.200
Croatia	Raw milk		0.001-0.124 Mean: 0.006	Bilandžić *et al*., 2016	0.006	0.142 (CIFOCO, 2017) 0.663 (FAOSTAT, 2017)	0.0122 - 0.0568
	UHT milk		0.002-0.021 Mean: 0.006				
Egypt	Raw milk	38	0.023-0.073 Mean: 0.0171	Amer and Ibrahim, 2010		0.113 (FAOSTAT, 2017)	0.0277
Ethiopia	Raw milk	100	0.028-4.98 Mean: 0.41	Gizachew *et al*., 2016	0.6498[b]	0.085 (FAOSTAT, 2017)	0.7891
		100	0.029-2.159 Mean: 0.69	Tadesse *et al*., 2020			
	Pasteurized milk	100	0.55-1.41 Mean: 0.97				
France	Raw milk	3.1	0.008-0.026 Mean*:0.0024	Boudra *et al*., 2007		0.184 (CIFOCO, 2017) 0.713 (FAOSTAT, 2017)	0.0063 - 0.0243

Table 22 (cont'd)

Greece	Pasteurized milk	79.6	0.005-0.05 Mean*:0.0124	Roussi *et al*., 2002	0.0206	0.624 (FAOSTAT, 2017)	0.1838
	Raw cow milk	64.3	<0.005- 0.055 Mean*:0.0342				
	Conventional, organic and kid's milk	46.5		Tsakiris *et al*., 2013			
India	Pasteurized milk	82	0.027–2.281 Mean:0.397	Sharma *et al*., 2019	0.0719[a]	0.291 (FAOSTAT, 2017) 0.322 (INDIASTAT, 2016)	0.2991 - 0.3310
	Raw milk	45.3	Mean: 0.018	Nile *et al*., 2016			
		100	0.001–3.8 Mean**: 0.016	Siddappa *et al*., 2012			
	UHT milk	66.6	n.d-2.1 Mean*:0.0608				
Indonesia	Fresh milk	90	0.024-0.449 Mean: 0.219	Sumantri *et al*., 2019	0.2312[a]	0.018 (FAOSTAT, 2017) 0.200 (Sumantri *et al*., 2019)	0.0594 - 0.6605
	Pasteurized milk	100	0.10-0.57 Mean: 0.244				

Table 22 (cont'd)

Iran	Raw milk	63.97	<0.01–0.41 Mean:0.028	Ghiasian *et al.*,2007	0.0461	0.147 (FAOSTAT, 2017)	0.0971
		100	0.041–0.065 Mean:0.053	Tajkarimi *et al.*,2007			
		56.7	0.05-0.35 Mean:0.103	Sefidgar *et al.*,2008			
		73	0.017–0.390 Mean:0.055	Kamkar *et al*., 2014			
		84	Mean:0.068	Rahimi *et al*., 2009			
		35	0.005–0.100 Mean:0.013	Habibipour *et al*., 2010			
		100	0.004- 0.113 Mean:0.04	Kamkar *et al*., 2011			
		54	0.001–0.116 Mean:0.057	Tajkarimi *et al*., 2008			
		80	0.011-0.321 Mean:0.066	Fallah *et al*., 2015			
		46	0.012-0.189 Mean:0.022	Fallah *et al*., 2016			
		100	0.05-0.10 Mean:0.027	Movassaghghazani and Ghorbiani, 2017			
	Pasteurized milk	87.3	< 0.005–0.120 Mean:0.04	Nejad *et al*., 2019			
		94.9	0-0.035 Mean:0.022	Barikbin *et al*., 2015			
		100	0.193-0.254 Mean:0.235	Azizi *et al*., 2008			
		100	0.019-0.126 Mean:0.075	Mohamadi Sani *et al.*,2010			
		84	0.011–0.063 Mean:0.021	Riazipour *et al.*,2010			
		100	0.179-0.25 Mean:0.23	Sefidgar *et al.*,2011			

Table 22 (cont'd)

		76.2	0.006-0.071 Mean:0.023	Mohammadi Sani *et al.*,2012			
		100	0.008-0.089 Mean:0.018	Karimi *et al*., 2007			
		100	0.009–0.064 Mean:0.027	Riahi-Zanjani and Balali-Mood, 2013			
		100	0.002–0.064 Mean:0.016	Sani and Nikpooyan, 2013			
		92	0.002–0.090 Mean:0.032	Hashemi, 2016			
		40	0.011–0.094 Mean:0.034	Rahimi *et al*., 2012b			
		67	0.022–0.098 Mean: 0.064	Ali Nia and Babaee, 2012			
	UHT milk	100	0.193-0.259 Mean:0.222	Azizi *et al*., 2008			
		100	Mean:0.066	Rahimi *et al*., 2009			
		53	0.021-0.087 Mean:0.052	Heshmati and Milani, 2010			
		45	Mean:0.0195	Mohamadi *et al*., 2010			
		62	0.006- 0.515 Mean:0.046	Fallah, 2010			
		92	Mean:0.046	Rahimi *et al*., 2012b			
Iraq	Milk (local)	60	0.002- 0.252 Mean:0.15	Al-Mossawei *et al*., 2016	0.0794[a]	0.0413 (FAOSTAT, 2017)	0.0468
	Milk (imported)	40	0.0- 0.097 Mean:0.042				

Table 22 (cont'd)

Italy	UHT milk	41.7	0.003-0.005 Mean*:0.0021	Santini *et al*., 2013	0.0060	0.1568 (EFSA, 2018) 0.626 (FAOSTAT, 2017)	0.0135 - 0.0538
		57.7	0.0007-0.0036 Mean:0.0016	Campone *et al*., 2018			
	Pasteurized milk	99.5	0.00085-0.0444 Mean:0.0035				
	Conventional and organic milk	60.3	0.009–0.026 Mean:0.016	Armorini *et al*., 2016			
	Raw milk	92	0.005-0.025 Mean*:0.0104	Visciano., *et al*.,2015			
		52.9	0.003-0.016 Mean*:0.004	Santini *et al*., 2013			
		12.3	0.004- 0.052 Mean:0.037	De Roma *et al*., 2017			
Japan	Raw milk	100	Mean:0.0073	Sugiyama *et al*., 2008	0.0081	0.1606 (FAOSTAT, 2017)	0.0186
	Pasteurized milk	99.5	0.001–0.029 Mean:0.009	Nakajima *et al*., *(*2004)			
Jordan	Raw milk	100	0.007–0.130 Mean:0.056	Omar, 2012	0.0611[a]	0.1124 (WHO GEMS, 2012)	0.0981
	Pasteurized cow milk	100	0.015-0.217 Mean:0.059	Omar, 2016			
	Fresh cow milk		0.01-0.130 Mean:0.069				

Table 22 (cont'd)

Kenya	Raw milk		<0.002-1.100 Mean:0.131	Lindahl *et al*., 2018	0.0946[a]	0.2216 (FAOSTAT, 2017) 0.437 (Ahlberg *et al*., 2018)	0.2994 - 0.5903
		100	0.015-4.563 Mean:0.29	Kuboka *et al*., 2019			
			0.00- 2.93 Mean and LOD: not reported	Langat *et al*., 2016			
		59	Mean:0.123	Ahlberg *et al*., 2018			
	UHT and pasteurized milk	29	Mean:0.074				
	Pasteurized milk		<0.002-0.740 Mean:0.126	Lindahl *et al*., 2018			
			0.008-0.210 Mean:0.055				
	UHT milk		0.007-0.0840 Mean:0.046				
			<0.002-0.470 Mean:0.058				
Kuwait	Fresh milk (local)		n.d- 0.069 Mean:0.019	Dashti *et al*., 2009	0.0200	0.1312 (FAOSTAT, 2017)	0.0374
	Fresh milk (imported)		n.d-0.063 Mean:0.021				

Table 22 (cont'd)

Lebanon	Raw milk	58.8	0.011–0.440 Mean:0.035	Daou *et al*., 2020	0.046	0.1712 (FAOSTAT, 2017)	0.1126
		73.6	0.0026-0.126 Mean:0.06	Assem *et al.,* (2011)			
	Pasteurized milk	88.8	0.001-0.117 Mean:0.031				
	Pasteurized and UHT milk	90.9	0.013–0.219 Mean:0.069	Daou *et al*., 2020			
	Raw and pasteurized cow milk	Not reported	Mean:0.022	Hassan and Kasssaify, 2014			
Malaysia	Liquid milk	33.3	Mean:0.009	Nadira *et al*., 2017	0.0263	0.0057 (FAOSTAT, 2017)	0.0021
	Fresh milk	4	0.020-0.142 Mean:0.092	Shuib *et al*., 2017			
Mexico	Fluid milk		0.1-1.27 Mean:0.495	Quevedo-Garza *et al*., 2020	0.495[a]	0.3261 (FAOSTAT, 2017) 0.373 (Carvajal *et al*., 2003)	2.3056 -2.638
Morocco	Pasteurized milk	88.8	0.001- 0.117 Mean:0.0186	Zinedine *et al*., 2007	0.0167	0.1448 (FAOSTAT, 2017) 0.175 (Zinedine *et al*., 2007)	0.0346 - 0.0418
	UHT milk	35	0.005-0.044 Mean:0.015	Alahlah *et al*., 2020			

Table 22 (cont'd)

Nigeria	Fresh milk	100	0.407-0.952 Mean:0.665	Susan *et al*., 2012	0.2169[a]	0.006 (FAOSTAT, 2017)	0.0185
	Fresh cow milk (nomadic)	80	0.011 - 1.354 Mean:0.531	Anthony *et al*., 2016			
	Fresh cow milk (commercial)	25	0.046 - 0.099 Mean:0.058				
	Raw milk		0.009-0.456 Mean:0.108	Oluwafemi *et al*., 2014			
Pakistan	Fresh milk	91.7	0.020 – 3.090 Mean:0.317	Asghar *et al*., 2018	0.1362[a]	Raw milk: 0.353 (Iqbal *et al*., 2017) 0.506 (FAOSTAT, 2017)	0.6867 - 0.9837
	Raw milk	37.5	Mean: 0.014	Hussain *et al*., 2010			
		71	0.004–0.845 Mean:0.151	Iqbal and Asi, 2013			
		80.95 (Lahore city)	0.69-100.04 Mean:17.38[b]	Muhammad *et al*., 2010			87.65-125.6
		64.9	LOD-0.346 Mean:0.111	Iqbal *et al*., 2017			
			0.3-1.0 Mean: 0.64	Akbar *et al*., 2019			
	Local shop, household and dairy farm milk	76.3 (all combined)	0.002-1.9 Mean:0.209	Sadia *et al*., 2012			
	Raw and processed	93	0.006–0.554 Mean:0.192	Ahmad *et al*., 2019			
	Milk	93	0.001–0.261 Mean:0.098	Ismail *et al*., 2016			
	UHT milk	70	LOD-0.303 Mean:0.086	Iqbal *et al*., 2017			

Table 22 (cont'd)

Palestine	Raw milk	85	0.020–0.080 Mean:0.029	Al Zuheir and Omar, 2012		0.626 (WHO GEMS, 2012)	0.0259
Portugal	Pasteurized and UHT milk	27.5	Mean:0.023	Duarte *et al*., 2013		0.151 (CIFOCO, 2017) 0.593 (FAOSTAT, 2017)	0.0497 - 0.1948
Rwanda	Raw milk		Mean:0.89[b]	Maier, 2018		0.054 (FAOSTAT, 2017)	0.683
Saudi Arabia	UHT milk	82	0.01-0.19 Mean: 0.058[a]	Abdallah *et al*., 2012		0.175 (FAOSTAT, 2017)	0.1453
Serbia	UHT milk		0.02-0.41 Mean:0.19	Kos *et al*., 2014	0.1369[a]	0.352 (WHO GEMS, 2012)	0.6891
	Pasteurized milk		0.06–1.20 Mean:0.366				
	Raw milk		0.005–0.90 Mean:0.19	Kos *et al*., 2014			
		85	<0.005–1.10 Mean:0.069	Milićević *et al*., 2017			
	Heat-treated milk	98.4	0.005–0.28 Mean:0.039				
		32.6	Mean:0.09	Tomasevic *et al*., 2015			
South Africa	Raw milk	87.1	0.01-2.85 Mean: 0.145[a]	Mulunda and Mike, 2014		0.147 (FAOSTAT, 2017)	0.3041
South Korea	Raw milk	48	0.002–0.08 Mean: 0.026	Lee *et al*., 2009		0.246 (WHO GEMS, 2012)	0.0914

Table 22 (cont'd)

Spain	UHT milk	68	–0.014 Mean:0.0097	Cano-Sancho *et al*., 2010		0.351 (Cano-Sancho *et al*., 2010) 0.493 (FAOSTAT, 2017)	0.0486 - 0.0683
Sudan	Raw milk	100	0.1 - 2.52 Mean:0.92	Ali *et al*., 2014	0.2520[a]	0.289 (FAOSTAT, 2017)	1.0391
		98.6	0.018-0.086 Mean:0.069	Suliman and Abdalla, 2013			
Syria	Raw cow milk	95	0.020–0.690 Mean:0.143	Ghanem and Orfi, 2009	0.2477[a]	0.258 (WHO GEMS, 2012)	0.9143
	Pasteurized milk	100	0.008–0.765 Mean:0.429				
Taiwan	Pasteurized milk	69.4	0.001-0.055 Mean*:0.0053	Peng and Chen, 2009	0.0079	0.082 (FAOSTAT, 2017)	0.0092
	Fresh milk	90.9	0.002-0.083 Mean*:0.0118	Lin *et al*., 2004			
Tanzania	Raw milk	83.8	0.026-2.007 Mean: 0.297[a]	Mohammed *et al*., 2016		0.107 (FAOSTAT, 2017)	0.4558
Thailand	Raw milk	100	0.05-0.197 Mean:0.068	Ruangwises and Ruangwises, 2009	0.0482	0.040 (FAOSTAT, 2017)	0.0278
	Pasteurized milk		0.004-0.293 Mean**:0.034	Suriyasathaporn and Nakprasert., 2011			

Table 22 (cont'd)

Turkey	Raw milk	21.1	0.011-0.1 Mean: 0.036	Sahin *et al*., 2016	0.05	0.482 (FAOSTAT, 2017)	0.3429
		53	0.025-1.01 Mean:0.153	Golge, 2014			
		86	0.001–0.030 Mean:0.0087	Ertas *et al*., 2011			
		17	0.005–0.300 Mean:0.083	KESKIN *et al*., 2009			
	UHT milk	58.1	n.d – 0.544 Mean:0.108	Unusan, 2006			
		67	0.01–0.63 Mean:0.067	Tekinsen and Eken, 2008			
		59	0.010–0.051 Mean:0.022	Gürbay *et al*., 2006			
	Pasteurized milk	100	0.005–0.080 Mean:0.060	Buldu *et al*., 2011			

* = mean calculated by taking the non-detect samples into account assuming the minimum value to be one-quarter of the LOD
** = mean calculated using the range
[a] = AFM1 levels exceeding EU regulatory limits of 0.05 µg/L in milk
[b]= AFM1 levels exceeding both EU limits (0.05 µg/L) and FDA regulatory limits of 0.5 µg/L in milk
ADD = Average daily dose
UHT milk = Ultra-high-temperature treated milk

As can be seen in Table 21, there is a wealth of studies measuring AFM1 levels in liquid milk in various forms, showing dramatically different results for AFM1 occurrence across the world as well as within the same country. Most countries had studies that showed at least a proportion of the milk having no detectable AFM1; and even among the detectable levels, most studies around the world showed AFM1 levels below the EU action level of 0.05 µg/L.

There were, however, several countries that had samples showing over the FDA action level of 0.5 µg/L AFM1: Algeria, Brazil, Ethiopia, Iran, India, Kenya, Mexico, Nigeria, Pakistan,

Palestine, Serbia, South Africa, Sudan, Syria, Tanzania and Turkey. In particular, one study in Pakistan (Muhammed *et al.*,2010) showed extraordinarily high AFM1 levels – up to 100 µg/L. If this measurement was accurate, then that would imply an aflatoxin B1 level in animal feed of about 1000-6000 µg/kg (van Egmond and Dragacci 2001): dangerously high for dairy animals and humans alike.

In addition to these occurrence data, we also compiled data on human intake rates of milk around the world, relying on multiple different sources and using the WHO GEMS database when no other sources provided information on a country-level basis. We used the exposure calculation equation in the Methods section to thereby determine average human exposure to AFM1 on a per-country basis: ADD, or average daily dose.

At the moment, we cannot compare our exposure estimates for AFM1 in each nation to any sort of nationally or globally accepted metric; as no tolerable daily intake (TDI) has been set for AFM1. Indeed, the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization and World Health Organization has not set TDIs for any of the aflatoxins, including AFM1; while it has set TDIs for other mycotoxins such as fumonisin, deoxynivalenol, T-2 toxin, and HT-2 toxin (JECFA 2016).

Table 22 shows the results of our literature search on AFM1 occurrence in powdered milk. Powdered milk is an important source of dairy (and protein) in the diets of many people around the world, where refrigeration is unavailable or unreliable, and/or where shelf-stable foods are commonly sold. Compared with liquid milk in its various forms, there are far fewer studies measuring AFM1 in powdered milk, and there is not more than one study per country in Table 2. Therefore, it is not entirely clear that the one study per country is representative of all areas of the country or accounts for seasonality of aflatoxin exposure in dairy animals (and therefore, AFM1

levels in powdered milk). However, powdered milk is a relatively homogenous food product, and these studies may in fact have found AFM1 levels that are taking dairy samples from across the nation at different times of year.

Table 23: Aflatoxin M_1 occurrence in powdered milk in different countries.

Country	% AFM1 (+) samples	Range (µg/L)	Mean (µg/L)	Reference
Argentina	80		0.013	Lopez *et al*., 2003
Brazil	100	0.33-0.81	0.61[b]	Sifuentes dos Santosal *et al*., 2015
China			0.016	Huang *et al*., 2014
Colombia	100	0.20–1.19	0.59[b]	Marimón Sibaja *et al*., 2019
Jordan	100	0.018-0.289	0.104[a]	Omar, 2016
Lebanon	35.7	0.0092–0.016	0.014	Assem *et al., (*2011)
Malaysia	3		0.021	Nadira *et al*., 2017
Morocco	100	0.015-0.039	0.026	Alahlah *et al*., 2020
Pakistan	28.1	0.0004- 0.179	0.065[a]	Iqbal *et al*., 2017
	37.5	0.0004- 0.278	0.090[a]	
Serbia			0.847[b]	Tomasevic *et al*., 2015
Sudan	95.5	0.22-6.9	2.07[b]	Elzupir and Elhussein, 2010
	100	0.01 – 0.85	0.29[a]	Ali *et al*., 2014
Syria	13 (1 sample)		0.012	Ghanem and Orfi, 2009
United States	40		0.096[a]	Kawamura *et al*., 1994

[a] = AFM1 levels exceeding EU regulatory limits of 0.05 µg/L in milk
[b]= AFM1 levels exceeding both EU (0.05 µg/L) and FDA regulatory limits of 0.5 µg/L in milk

Of these studies of powdered milk, only the Sudan study (Elzupir and Elhussein 2010) show unusually high AFM1 levels. All other countries except Brazil, Colombia and Serbia have levels below the FDA limit of 0.5 µg/L, and most have levels exceeding the EU limit of 0.05 µg/L. Exposure calculations were not done, as there were no available data on consumption rates of powdered milk for any nation.

4. Discussion

The goal of this work was to estimate aflatoxin M1 exposure in human populations in different nations of the world, assuming that the primary food source of AFM1 was milk. We have calculated these exposures as average daily dose per capita, per nation, for liquid milks (Table 21). However, we acknowledge that additional exposure to AFM1 can be present due to consumption of other dairy products such as cheese, butter, and yogurt, which we have not covered in this review. We cannot yet state whether these exposures in different world populations are likely to cause adverse human health effects because no nation and no international standard-setting institution (such as JECFA) has yet set a tolerable daily intake for AFM1. Nonetheless, based on the available evidence of AFM1-induced adverse health effects from *in vivo* and *in vitro* toxicological studies, exposure to this mycotoxin should be kept as low as reasonably achievable.

A plethora of studies measure aflatoxin M1 levels in liquid milk around the world; sometimes multiple studies from the same country. The liquid milk types included: raw milk, pasteurized milk, "fresh milk" (the studies did not define what this meant), ultra-high-temperature (UHT)-treated milk, conventional milk, and organic milk. The nations that we identified to have AFM1 levels occasionally (and sometimes dramatically) exceeding the FDA action level are primarily in sub-Saharan Africa and South Asia. In particular, milk samples from Pakistan showed occasional high excursions (100 µg/L AFM1), which may imply that the crops used to make that feed, which humans might also consume (such as maize and various types of nuts and seeds), may have high AFB1 levels – potentially in the thousands of µg/kg – that could harm human health. If moldy foodstuffs were deliberately being diverted to animal feed rather than human food, then indeed, human health would be somewhat spared from high AFB1 exposure in these regions. By comparison, milk in the US consistently has AFM1 levels below the FDA action level of 0.5 µg/L.

Far fewer studies are available on AFM1 levels in powdered milk; however, we did find 13 studies, representing as many nations, measuring AFM1 in this foodstuff. There were few extremely high excursions above FDA action levels; and, if the powdered milk were blended with water, it is likely that most of these samples would result in overall AFM1 concentrations below this action level. However, the AFM1 in most powdered milk samples would exceed the EU limit. It is not possible for us to do an exposure assessment of AFM1 from powdered milk sources at this point, as there were no publicly available data on consumption levels of powdered milk for any nation.

Future work in this area would focus on combining these exposure calculations with reliable health effects data on AFM1, to assess risks to human populations worldwide. To do so, it is important to find reliable toxicological data surrounding aflatoxin M1, to derive the most reliable dose-response information to contribute to this risk assessment. A concern is that there will be a significant challenge in finding reliable studies examining health effects of AFM1 that are independent of health effects caused by its parent compound, AFB1.

One limitation of past studies attempting to link adverse health effects to AFM1 is that AFM1 is, in fact, a biomarker of AFB1. Importantly, it is a metabolite that indicates that part of the AFB1 did *not* become biotransformed to its carcinogenic form: AFB1-8,9-exo-epoxide, which binds to DNA and liver proteins, and can initiate cancer or cause liver dysfunction (Groopman *et al.,*2008). Therefore, unless the AFM1 was directly administered to laboratory animals or directly consumed by humans in epidemiological studies (in the absence of consuming AFB1-contaminated foods), it is not possible to use AFM1 levels in urine or milk as an indicator of adverse effects caused directly by AFM1. Any observed adverse effects in those cases could instead be a result of AFB1 exposure, for which AFM1 may serve as a biomarker. Nonetheless, such work is important in

informing the setting of AFM1 standards around the world and to evaluate whether the standards set are practically achievable or not, especially in developing countries.

Although mycotoxin contamination in food occurs in every nation, it is more prevalent in the developing countries where the climate and storage conditions favor the fungal growth, there is lack of advanced agricultural practices and strict food regulations (Shephard 2008). Several *in vivo* and *in vitro* studies suggest that exposure to AFM1 in milk may play a critical role in aflatoxicosis. Therefore, the occurrence of AFM1 in milk and milk products, its potential toxic effects, and resistance to heat treatments and pasteurization are critical public health issues.

In summary, several nations in South Asia and sub-Saharan Africa had AFM1 levels in milk that substantially exceeded US and EU regulatory limits for AFM1, indicating potential risk to humans in those regions who consume large amounts of milk. Of particular concern are populations of children, who may consume relatively more milk and may be more vulnerable to the potential adverse effects from AFM1 exposure. Understanding AFM1 occurrence and exposure is also important in identifying geographic regions where AFB1 levels in staple food and feed crops are high enough to cause concern for human and animal health.

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